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Function of Cancer Associated Protein Translin

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School of Biological Sciences

Bangor University

Ph.D. thesis 2012

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Abstract

Translin and its binding partner protein Translin-associated factor-X (TRAX) have been implicated in a diverse range of cellular processes. Translin has been demonstrated to bind to both DNA and RNA and appears to be involved in the recognition of specific sequences associate with breakpoint junctions of chromosomal translocations linked with the development of some human cancers. More recently, Translin and TRAX have been found to make a heterocomplex known as C3PO which has been implicated in passenger strand removal in the RNAi pathway and tRNA processing. Translin is conserved in the fission yeast and in this study we used this facile model system to further investigate the biological function of Translin. Initial work confirmed previous findings that demonstrated loss of Translin function alone has no measureable negative affect on fission yeast cells. However, we addressed the hypothesis that Translin functioned in a redundant pathway with the RNAi pathway component Dicer and found that when Dicer and Translin are disrupted there are enhanced levels of genome instability which are not due to failures in the DNA damage response. Further investigation demonstrated that this was due to an enhanced failure in the function of centromeric heterochromatin, which is regulated in part by the RNAi pathway. Here we present a model demonstrating that Translin is the key regulator of a previously inferred argonaute-dependent, Dicer-independent regulator of centromeric heterochromatin function and chromosome stability.

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Abbreviations

AML: acute myeloid leukaemia

AP: apurinic or apyrimidinic

AT: Ataxia Telangiectasia

BDNF: Brain-derived neurotrophic factor

BER: base excision repair

bp: base pair

CML: chronic myelogenous leukaemia

C3PO: component 3 promoter of RISC

DAPI: 4'-6-diamidino-2-phenylindole

DNA-PK: DNA-dependent protein kinase

Dhj: double Holliday junction

DSB: double strand break

DSBR: double strand break repair

dsDNA: double-stranded DNA

ssDNA: single-stranded DNA

EDTA: ethylenediaminetetraacetic acid

EMM2: Edinburgh minimal medium2

5-FOA: 5-Fluoroorotic Acid

HDAC: Histone deacetylase

HEK: human embryonic kidney

HJ: Holliday Junction

HR: homologous recombination

HU: hydroxyurea

IR: inverted repeats

kb: kilobase

kDa: kilo Dalton

LB: Luria-Bertani media

LiAc: Lithium Acetate

LTR: long terminal repeat

Mb: megabase

ME: malt extract

MEF: mouse embryonic fibroblast

MMC: mitomycin C

MMR: mismatch repair

MMS: methyl methanesulphonate

mRNA: messenger RNA

mRNP: ribonucleoprotein

NB: nitrogen base

NBL: nitrogen base liquid

NER: nucleotide excision repair

NHEJ: non-homologous end joining

ORF: open reading frame

PCR: polymerase chain reaction

PEG: polyethylene glycol

rDNA: ribosomal DNA

RDR: recombination-dependent replication

RdRP: RNA- dependent RNA polymerase

RIPA: radioimmunoprecipitation assay

RISC: RNA-induced silencing complex

RITS: RNAi-induced transcriptional silencing

RNAi: RNA interference

RNA polIII: RNA polymerase III

rRNA: ribosomal RNA

r.p.m.: revolutions per minute

SDS: Sodium Dodecyl Sulfate

siRNA: small interfering RNA

SPA: synthetic sporulation media

SSB: single-strand break

ssDNA: single-stranded DNA

TB-RBP: testes brain RNA binding protein

TBZ: thiabendazole

TF: transcription factor

TB-RBP: Testis –brain –RNA binding protein

TRAX: Translin associated factor-X

TSA: Trichostatin A

tRNA: transfer RNA

UTR: untranslated region

UV: ultra-violet

WCE: whole cell extract

YE: yeast extract

YEA: yeast extract agar

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Chapter 1

Introduction

1.1 Cancer

One eighth of all worldwide deaths are caused by cancer (Michael *et al.*, 2009). It causes more than 100 different types of disease within a wide range of human organs (Michael *et al.*, 2009; Jeffords and Irminger-Finger, 2006). Cancer is considered to be a complicated set of diseases that are characterised by changes in genome sequences and structure (Michael *et al.*, 2009; Hanahan and Weinberg, 2011). A key feature of cancers is the uninhibited multiplication of cells (neoplasms), which can invade normal tissue boundaries and transfer to distinct organs, establishing secondary tumours in a process called metastasis (Michael *et al.*, 2009). Many genes have been implicated in tumorigenesis and cancer progression (Futreal *et al.*, 2004), and oncogenic change can occur due to the activation of proto-oncogenes or the deactivation of tumour suppressor genes via mutations or deletions (Peltomäki, P. 2011; Beerenwinkel *et al.*, 2007; Calasanz and Cigudosa, 2008). Over a prolonged period of time, these alterations can result in the unlimited proliferation of cells and/or a decrease in level of cell death (Beerenwinkel *et al.*, 2007). Some genetic changes can be inherited and cause a predisposition to cancer. Throughout the development of malignant tumours, the change from a normal cell to a metastatic cancerous cell is characterised by numerous physiological changes that are divided into a number of groups, including: avoiding growth suppressors (anti-growth signals), evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and lastly, tissue invasion by metastasis (Hannah and Weinberg, 2011). Furthermore, defects in DNA metabolism or DNA repair in normal cells are strong factors

in causing chromosome instability and the predisposition toward a variety of cancer types, for example, bladder, breast, lung, and colon cancers (Bartkova *et al.*, 2005; Harrison *et al.*, 2002).

In addition to intracellular events contributing to DNA damage levels, the alteration of a normal cell into a cancerous cell is also caused by changes that occur due to external or environmental factors, for example, chemical carcinogens, ionizing radiation (x-rays and UV light), viruses, and even chemotherapy (Venkatesan *et al.*, 2006; Ashworth *et al.*, 2011).

1.2 Genomic instability

Threats to genome stability range from point mutations to gene amplifications, deletions, chromosomal rearrangements, and changes in the number of chromosomes, which can lead to loss or gain of whole chromosomes (Aguilera and Gomez-Gonzalez, 2008). Therefore, it is widely accepted that these abnormalities of number and structure of chromosomes, or genetic mutations and epigenetic changes are a hallmark of tumours and are a major force in the development of cancer (Gordon *et al.*, 2012; Weberpals *et al.*, 2011; Lord and Ashworth, 2012). Chromosome instability arises due to defects in DNA metabolic processes, such as DNA replication, telomere maintenance, and/or DNA repair in normal cells, all of which protect the genome from changes (Bartkova *et al.*, 2005; Harrison *et al.*, 2002; Anderson, 2001). If damaged DNA is not repaired this can eventually lead to genomic instability (Lombard *et al.*, 2005). In the case of DNA replication failure, genetic recombination can occur, which if mediated via an incorrect partner it can result in chromosomal translocations and other larger structural changes. These alterations can change cell behavior and cause diseases development (Lord and Ashworth, 2012). Maintaining genome stability is fundamentally important for the correct functioning of all

cells. Genome instability is also implicated as a primary cause of cellular aging, as indicated by the accelerated cellular aging associated with defects in genome maintenance programs in a variety of organisms (Faggioli *et al.* 2011; Burhans *et al.*, 2007). Cells are continually at risk of cytotoxic and/or mutagenic events. Many of these events occur often during the lifetime of a cell due to exposure to a variety of stressing agents, which arises from both external and internal factors, for example, metabolic byproducts and radiation, (Reinhardt and Schumacher, 2012). Specific factors causing genome instability include reactive oxygen species, the impaired fidelity of DNA replication and mitotic chromosome segregation, defects in DNA repair systems, and the inactivation of cell cycle checkpoints, which normally arrest proliferation in response to DNA damage (Kopnin, 2006). In order to prevent eukaryotic genome damage, many processes that take place during cell proliferation must be tightly controlled and coordinated. The replication of DNA is particularly prone to failures despite being a relatively high fidelity process. Therefore, many of the abnormalities that can occur in chromosomes arise due to defects in the replication of chromosomes (Branzei, 2005; Jones and Peterman, 2012). Correctly functioning checkpoints and repair pathways are both necessary to ensure that replicative errors are appropriately dealt with.

1.3 Gross chromosomal rearrangements

Chromosomal mutations occur as a result of aberrations in the sequences or structure of the chromosomes, either with no apparent cause or as a result of exposure to chemicals or radiation. Sandberg (1966) examined solid tumours in a number of patients and observed that the chromosome structure exhibited abnormalities in almost all types of cancer. Highly advanced tumours and malignant discharge were especially associated with substantial chromosome aberrations, including hypotetraploid numbers of chromosomes and excessive

chromosome restructuring (Nowell, 2007). Chromosomal number alteration in the germ cells is also a significant cause of developmental disorders, such as Down's syndrome. There are four main types of genetic structural aberrations, namely, deletions – manifested as loss of DNA; duplications – manifested as reproduction of DNA segments; inversions – the chromosomes exhibited alterations in the orientation of DNA chain segments, without loss of DNA; and chromosomal translocations (Mazumdar, 2007).

1.4 Chromosomal translocations

Chromosomal translocations result from the interchange of entire chromosome arms between nonhomologous chromosomes by incorrect repair of chromosomal breaks including double-strand breaks (DSBs) which are repaired incorrecion (Tucker, 2010). In some cases, chromosomal translocations can sometimes lead to gene separation, gene activation or the development of new genes which incorporate fusion proteins (Willman and Hromas, 2006). Chromosomal translocations are associated with a variety of cancers, such as haematologic malignancies and sarcomas (mesenchymal tumors) (Alpan, 2006; Rabbitts and Stocks, 2003). The translocation of chromosomes can lead to the generation of oncogenes via the activation of proto-oncogenes, by aberrant activation of expression (Agarwal *et al.*, 2006; Rabbitts and Stocks, 2003). The ends of the break might be rejoined, but may have additional or missing DNA sequences at the joint (Tucker, 2010). Many kinds of cancer are highly associated with chromosomal translocation (Hakim, 2012). Translocations and rearrangements are considered central to the creation of tumours but their origins are still poorly understood (Hakim, 2012). The translocation between chromosome t(9;22)(q34;q11), which is considered to be the first consistent chromosomal translocation found in human cancer, results in the Philadelphia chromosome (Stefanachi, 2012; Calasanz and Cigudosa, 2008). The symbols t(9;22)(q34;q11) indicate a

translocation between chromosome 9 and 22 (Fielding, 2011; Mitelman *et al.*, 2007), which causes chronic myeloid leukemia (CML) by the creation of a BCR-ABL fusion protein (Yeung and Hughes, 2012).

Chromosomal translocation can be linked to normal cell processes, such as immunoglobulin or T-cell-receptor gene rearrangement (Hakim, 2012), when the breakdown of chromosomes occurs inter-chromosomally, instead of as normal intra-chromosomal translocation. There are two main kinds of chromosomal translocations, reciprocal (non-Robertsonian) and Robertsonian (Figure 1.1). In addition, translocations can be balanced, having an equal exchange of material with no substantial net gain or loss of nuclear DNA, or unbalanced, in which the exchange of chromosome material is not reciprocal, resulting in the addition or loss of genes. Reciprocal (non-Robertsonian) translocations are frequently an exchange of material between different chromosomes (Traversa *et al.*, 2010). These mechanisms have been detected in a variety of tumours, such as leukemia and lymphoma, and they have been associated with the etiology of these cancers (Gollin, 2007). Nonreciprocal translocation (Robertsonian translocation) represents the most common human structural chromosomal abnormalities (Hamerton *et al.*, 1975). These include breakpoints close to the centromere of two acrocentric chromosomes (Traversa *et al.*, 2010). These types of chromosomal translocations are seen most often in the acrocentric chromosomes: 13,14,15, 21, or 22.

In addition, chromosome translocation can occur when the normal replication of the chromosome is disturbed (Mirikin and Mirikin, 2007). In humans, yeast, and mice, the translocation of chromosomes can happen when the DNA repair process is interrupted (Rabbitts and Stocks, 2003).

Translin is a protein which has a high ability to bind to the single-stranded DNA sequence motifs 5'-ATGCAG-3' and GCCC(A/T)(G/C)(G/C)(A/T), which flank several major breakpoint junctions of chromosomal translocations in several human lymphoid neoplasms, including and involving: 1p32, 3q27, 5q31, 8q24, 9q34, 9q34.3, 10q24, 11p13, 14q11, 14q32, 14q32.1, 17q22, 18q21, 19p13, and 22q11 (Aoki *et al.*, 1995; Kasai *et al.*, 1992; Kasai *et al.*, 1997).

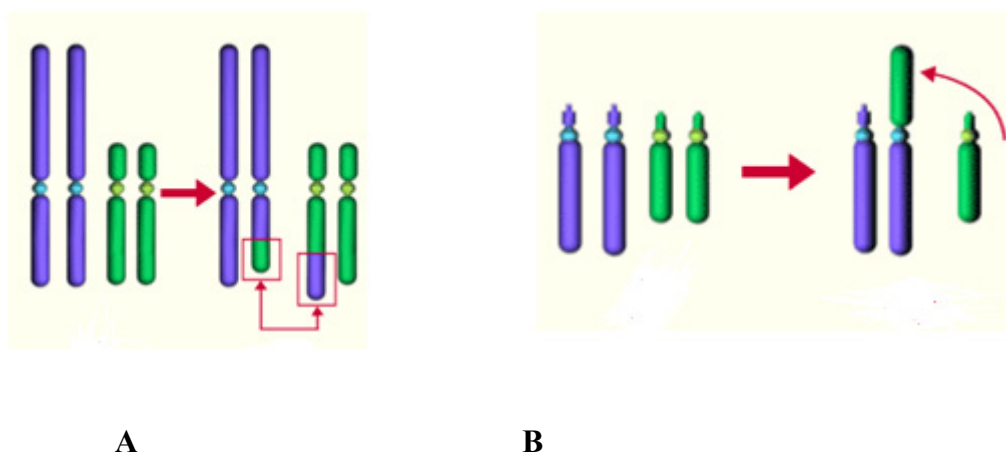


Figure 1.1 Reciprocal and non-reciprocal translocation

A. Reciprocal translocation

Reciprocal translocation is an exchange of material between two broken chromosome pieces from two non-homologous chromosomes joining together and creating a new hybrid chromosome.

B. Non-reciprocal translocation

A non-reciprocal translocation entails the loss of the short arms by two chromosomes and the fusion of the q-arms, which causes two heterologous chromosomes to unite through the process of centric fusion

These observations led to the hypothesis that translin may play a role in mediating chromosomal rearrangements, including translocation (Gajecka *et al.*, 2006). (see Section 1.7 for further details).

1.5 DNA damage

Specific DNA repair mechanisms have evolved to repair different types of DNA damage, and to maintain genomic integrity (Wu and Yu, 2012). DNA damage can be produced from exogenous factors, for example, ionizing radiation (IR), ultraviolet (UV) radiation, (Wu and Yu, 2012) and other risk factors, such as chemical carcinogens, or from endogenous factors for instances stalled replication forks due to replication stress and byproducts of metabolism. These intrinsic and extrinsic factors can take the form of base modifications, strand breaks, interstrand cross-links, and other more complex lesions (Ciccia and Elledge, 2010; Kim, 2012; Hoeijmakers, 2001). Cells have developed a number of defence mechanisms, such as DNA repair and cell-cycle check-point processes, in order to withstand and cope with DNA damage.

1.6. DNA repair mechanisms

The genetic information in all living organism has to be preserved and corrected for transmission to the next generation (Lans *et al.*, 2012). To maintain genome stability, chromosomal DNA is under constant molecular surveillance. To maintain the integrity of the genetic information, DNA repair must be a high fidelity process, so a number of repair mechanism have evolved to keep genetic changes at a suitably low level (Kim, 2012). It is essential for cells to be able to repair DNA damage, which can otherwise lead to a rise in cancer incidence as well as multiple metabolic changes(Christmann *et al.*, 2003). Of all the forms of DNA damage, DSBs are probably the most hazardous to the integrity of the genome (Polo and Jackson, 2011; Jones and Petermann, 2012). However, double-strand breaks (DSBs) can have positive results when they develop during certain specialised

processes, such as the development of the immune system and homologue fusion in meiosis, which require the restructuring of the genome sequence (Wyman and Kanaar, 2006).

Depending on the kind of damage and mechanism involved, there are at least five main, partly overlapping damage repair pathways operating in mammals, including base-excision repair (BER), nucleotide-excision repair (NER), mismatch repair, homologous recombination (HR), and non-homologous DNA end-joining (NHEJ) (Moraes *et al.*, 2012).

1.6.1 Non-homologous DNA end-joining

The balancing of DSB repair activities appears to be critical to the genetic stability of cells. The two major pathways for the repair of DSBs are non-homologous DNA end-joining (NHEJ) and homologous recombination (Lord and Ashworth, 2012). Failure to balance homologous recombination and NHEJ pathways and restrict them to the correct cell cycle stage leads to the acquisition and persistence of mutations and translocations that can result in cell death and consequently to tumorigenesis (Rass *et al.*, 2012; Polo and Jackson 2011). Elevated levels of repair are essential for viability and can inhibit the apoptotic pathway enabling a cell with badly damaged DNA to survive with mutated/mis-repaired DNA (Seedhouse *et al.*, 2004). Non-homologous end joining (NHEJ) occurs primarily during the G1 phase of the cell cycle. Furthermore, when substantial homology is absent, NHEJ connects separated DNA ends, which can cause the development of abnormalities, such as an increase or decrease in the number of nucleotides, related to the type of the disrupted DNA ends as well as the manner in which they are treated before being joined (Lans *et al.*, 2012).

There are a number of essential proteins which take part in the canonical NHEJ, such as the Ku70/Ku80 DSB-binding heterodimer, which creates a ring-like structure particularly

around the disrupted DNA ends of chromosomal translocations to prevent their deterioration. where binding of DNA ends by KU70/ KU80 arrange the action of others repair factors by recruiting the phosphatidylinositol 3-kinase DNA-PKcs/ PRKDC (Lans *et al.*, 2012; Grabarz *et al.*, 2012; Doherty and Jackson, 2001; Smith and Jackson, 1999; Walker *et al.*, 2001). Thus, the additional repair proteins are activated when ARTEMIS/DCLRE1C nuclease is phosphorylated by the DNA protein kinase catalytic subunit, then the DNA ends are joined by the activity of DNA ligase IV and its cofactor XRCC4 to seal the break (Kasperek and Humphrey, 2011; Nasiri *et al.*, 2012; Lans, *et al* 2012; Martin and MacNeill, 2002). The loss or addition of a few nucleotides is the main drawback of this repair method. Therefore, it is considered error prone (Lans *et al.*, 2012, Weterings and David, 2008).

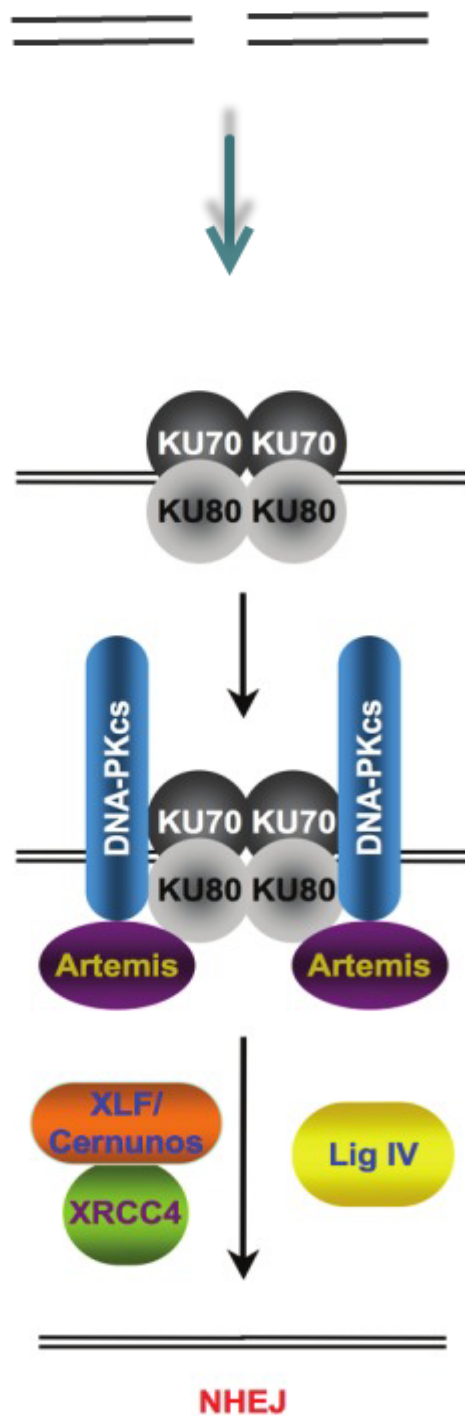


Figure 1.2 Non-homologous end joining.

After the formation of DSBs, Ku70/80 heterodimer associates with the ends of the broken DNA, non compatible DNA termini need to be processed by promotes the recruitment of DNA-PKcs and Artemis to be ready for ligation by the complex Cernunnos-XLF/XRCC4/Ligase IV complex at the final step (adapted from Grabarz *et al.*, 2012).

1.6.2 Homologous recombination repair

Chromosomal DSBs are considered the most severe type of DNA damage (Khanna and Jackson, 2001; Zhu *et al.*, 2012) and they can occur spontaneously and may happen as a result of external agents, including ionizing radiation, UV radiation, chemicals or endogenous agents, such as free radicals or mechanical stress on the chromosome (Bordeianu *et al.*, 2011; van Gent *et al.*, 2001; Neale and Keeney, 2006; Shrivastav *et al.*, 2008; Krogh and Symington, 2004; San Filippo *et al.*, 2008). HR repair can occur in S and G2 phases of the cell cycle because it requires a homologous sequence (a sister chromatid) as a template to copy the DNA elements. This pathway usually results in error-free repair of a DSB (Zhu *et al.*, 2012; Henrique Barreta *et al.*, 2012; Raji and Hartsuik, 2006; Shrivastav *et al.*, 2008) therefore, the repair will be accurate if the repair template is perfectly homologous (Shrivastav *et al.*, 2008). In cases where the template is not a sister chromatin, the repair templates are often not completely homologous, and this can lead to non-reciprocal exchange of genetic information from the donor locus to the recipient locus. This is a process called gene conversion (Shrivastav *et al.*, 2008; Grabarz *et al.*, 2012). The classical DSB repair model for HR is initiated with the nucleolytic resection of the DNA DSB ends to generate a single-stranded DNA (ssDNA) overhang with 3' OH ends. In mammals, this process of resectioning is mediated by the activities of the heterotrimeric MRN complex (in *S. pombe* Rad32–Rad50–Nbs1) together with CtIP, BLM, EXO1 and DNA2 (Fig. 1.3) (Suwaki *et al.*, 2011; Filippo *et al.*, 2008). Consequently, the single-stranded DNA binding protein RPA (in *S. pombe*, *rad11*) coats the 3' tail (Raji and Hartsuiker, 2006). Then the resulting 3' ssDNA loads Rad51 and invades a homologous duplex and serves as primer for copy synthesis, (Grabarz *et al.*, 2012; Raji and Hartsuiker, 2006; Krogh and Symington, 2004). RPA is eliminated from ssDNA by the

BRCA2/PALB2 complex permitting (Grabarz *et al.*, 2012) ssDNA tails to be coated with Rad51 (called Rhp51 in *S. pombe*). Rad51 plays a central role in all three phases of HR strand invasion: presynapsis, synapsis and post-synapsis (Krejci *et al.*, 2012; Akamatsu *et al.*, 2007). In the presynaptic phase, Rad51 is loaded at the break of a ssDNA and this loading, which depends on RPA, Rad52, BRCA2 and the Rad51 paralogs, results in nucleate formation of a nucleoprotein filament (Krejci *et al.*, 2012; Suwaki *et al.*, 2011). In the synaptic phase a heteroduplex, displacement DNA (D-loop) is generated by a physical association between the invading DNA strand and homologous duplex DNA template; in a process called strand invasion, which is stimulated by the interaction between Rad51 and Rad52 (Figure 1.3) (Krejci *et al.*, 2012; Suwaki *et al.*, 2011; Filippo *et al.*, 2008). After that, and during the post-synaptic phase, extension of D-loop generates a double Holliday junction (dHJ) by Rad52 mediation, where, the 3' single-stranded end is used as a primer for DNA synthesis to recover any missing DNA sequences because the removal of Rad51 gives DNA polymerases access to start the DNA synthesis reaction repair (Tan *et al.*, 2003; Krejci *et al.*, 2012; Suwaki *et al.*; Svendsen and Harper, 2010). HJ branch migration is produced by Rad54, then HJ resolution is mediated by GEN1 or the SLX1/4 complex or the Mus81-Eme1 complex, and produce crossover or non-crossover. Furthermore, the another pathway by HJ dissolution is mediated by Sgs1/Top3 helicase–topoisomerase complex (BLM/TOP3a in mammals) (Suwaki *et al.*; Svendsen and Harper, 2010).

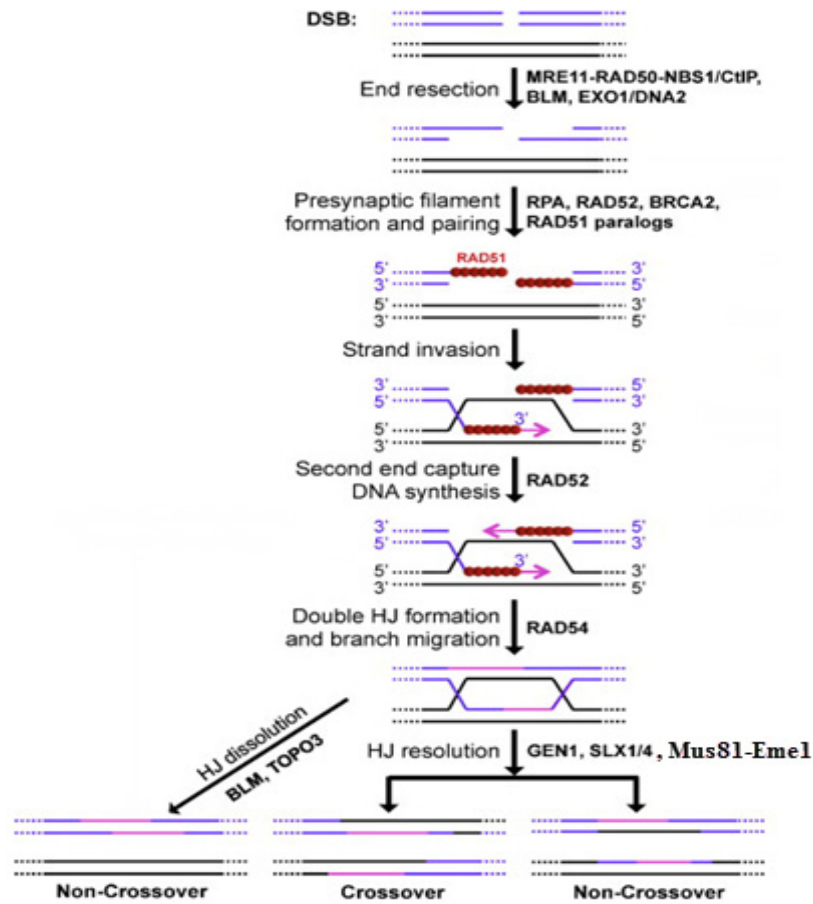


Figure 1.3 Schematic diagram of a double for DSB repair through homologous recombination.

The broken ends are recognised and processed by the activities of the MRE11–RAD50–NBS1 complex and CtIP to make single-stranded 3'overhangs. The resection is extended by additional helicase/nuclease activities including EXO1, BLM and DNA2. This process exposes ssDNA tails. Loading RAD51 on single-stranded DNA tails generated Rad51-ssDNA filament. The process of RAD51 loading depends on RPA, RAD52, BRCA2 and the RAD51 paralogs. This resulting undamaged homologous DNA sequence, which acts as a template DNA for DNA synthesis repair, is recognised and aligned by the formed Rad51 nucleoprotein filament. Next, a strand invasion Holliday junction (HJ) is formed by RAD52 meditation. HJ branch migration is promoted by RAD54. Then HJ resolution is provided by GEN1 or the SLX1/4 or the Mus81-Eme1 complex, and rejoining occurs with the formation of recombined DNA molecules. On the other hand, HJ desolution can be promoted by a BLM/TOP3 complex that leads to non-crossover products (adapted from Suwaki *et al.*, 2011).

1.7. Translin and TRAX

Translin is a highly conserved protein in eukaryotes, including humans (Jaendling and McFarlane, 2010). It is a recently identified nucleic acid binding protein that appears to be involved in the recognition of specific consensus sequences and repeated single-stranded microsatellite (GT) DNA and it associates with breakpoint junctions of some chromosomal translocations linked to the development of some human cancers, such as leukemia (Aoki *et al.*, 1995; Laufman *et al.*, 2005). Translin forms an octameric toroid (Figure 1.4) and has been postulated to be involved in diverse biological processes for example, DNA repair, regulation of cell growth, mRNA processing (Tian, 2011; Jaendling *et al.*, 2008; Ksai *et al.*, 2004), recombination (Aoki *et al.*, 1995), and/or telomere stability (Jacob *et al.*, 2004) by tightly binding to a single-stranded DNA or RNA (Tian, 2011). Under specific conditions, Translin has been found to localize inside the nucleus (Tian, 2011). Translin can also bind to G-rich DNA and single-stranded RNA sequences (Aoki *et al.*, 1995; Laufman *et al.*, 2005). In addition, it has a strong ability to bind to d(GT) and d(TTAGGG)_n repeats which has been proposed to facilitate a role in recombination repair of microsatellites and telomeres (Laufman *et al.*, 2005; Yu and Hecht, 2008) although no conclusive proof of this has been demonstrated. Translin binding sequences have also been identified in other cancer-associated translocation breakpoints (Wei *et al.*, 2003; Kanoe *et al.*, 1999; Hosaka *et al.*, 2000; Atlas *et al.*, 1998; Abeysinghe *et al.*, 2003) (see section 1.7.2). Chromosomal translocations cause many genetic changes commonly found in human neoplasms and are also associated with carcinogenesis (Weinstock *et al.*, 2006; Aplan, 2006). Thus, it was hypothesized that Translin might be involved in mediating chromosomal rearrangements, including translocations (Gajecka *et al.*, 2006; Gajecka *et al.*, 2006). Translin is the human orthologue of mouse testis-brain-RNA binding protein (TB-RBP) (Wu *et al.*, 1997). TB-RBP/ Translin also binds to DNA sequences found in

meiotic recombination hot spots, potentially implicating it in broader functional roles (Pascal *et al.*, 2001).

Trax, a Translin binding protein, first characterized as a Translin-like protein, was identified by yeast two hybrid assays (Aoki *et al.*, 1997) and by co-immunoprecipitation (Wu *et al.*, 1999). It has homology to Translin, but no intrinsic DNA binding activity (Aoki *et al.*, 1997). Trax has been associated with a structure known as ‘leucine zipper’, due to the fact that it is composed of a bipartite nuclear targeting motif and a heptad repeat of water repellent amino acids. This composition has been argued to play a significant role in the development of Trax homodimers or heterodimers with Translin (Aoki *et al.*, 1997). As for Translin, the biological function of Trax is unclear although recent work has implicated both in RNAi and tRNA processing (see below; Li *et al.*, 2012; Liu *et al.*, 2009). However, in the mouse Translin and Trax form DNA/RNA binding complexes isolated from the testes, suggesting they act at some stage of spermatogenesis (Wu *et al.*, 1999).

There are some studies that have suggested that Translin and Trax have a role in RNA metabolism and, Translin and Trax have recently been implicated in the RNA interference (RNAi) pathway and tRNA processing (see below). Some studies have indicated that Translin functions as an RNA binding protein that selectively identifies many meiotically expressed mRNAs and a non-coding RNA (Cho *et al.*, 2005).

Furthermore, in fission yeast Translin has been linked to the regulation of RNA metabolism, rather than DNA metabolism because of the higher affinities of Translin for RNA [GU]_n and [GUU]_n repeats (Laufman *et al.*, 2005). In addition to this, in brain and testis, Translin has been implicated in mRNA transport and/or stabilization and regulation of mRNA translation by its ability to bind to Y- and H-sequence of 39 untranslated regions

(UTRs) of target mRNA (Jaendling and McFarlane, 2010; Yu and Hecht 2008). Translin has also been reported to potentially stabilise a specific miRNA in germ cells (Yu and Hecht, 2008).

Translin and Trax has are primarily in the nuclei and in mouse neuronal dendrites, and they may have a role in neuronal function and development (Millar *et al.*, 2000; Muddashetty *et al.*, 2002). One of the interestingly roles of Translin and Trax in neurons cells is the ability of them to target mRNA of Brain-derived neurotrophic factor, BDNF, in its exon that contains a specific Translin/Trax-binding region. Mutation of the binding site of Translin and Trax within BDNF has been linked to human neurological disorders (Jaendling and McFarlane, 2010) this demonstrates a function for Translin and Trax in nervous system function and development (Jaendling and McFarlane, 2010; Millar *et al.*, 2000).

Recent biochemical analysis has shown that *Drosophila* Translin and Trax dimer make a ribonuclease activity complex called C3PO (component 3 promoter of RISC), where both of them are essential for the whole activity of RNA-induced silencing complex (RISC) and they have been considered as a crucial of the RNAi machinery (see below for further details of RNAi pathways) (Liu *et al.*, 2009; Tian *et al.*, 2011). The C3PO complex is Mg²⁺-dependent endonuclease and activates RISC by facilitating endonucleolytic cleavage of the passenger strand of the duplex siRNA, in addition to elimination of cleaved passenger strand, this process allows the guide strand to target cognate mRNA through Ago2 (Ye, 2011; Tian, 2011; Liu *et al.*, 2009). More recently, Translin and Trax have been implicated in tRNA processing, and it is proposed that this link to such a fundamental component of gene expression accounts for the apparent varied biological functions of Translin and Trax (Li *et al.*, 2012).

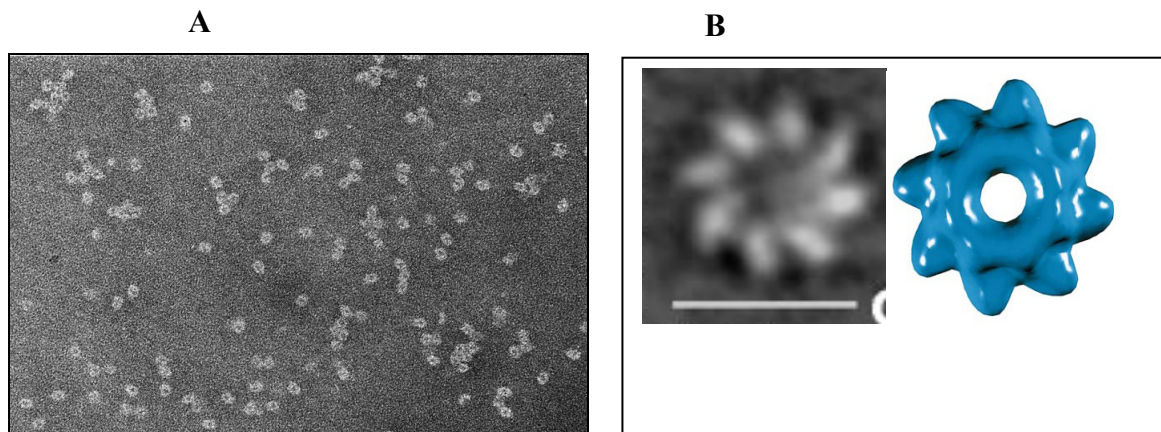


Figure 1.4 Electron micrograph of the Human Translin Protein in octameric toroidal form.

A. An electronic microscope image of native Translin.

In its original state, Translin is a circular octamer. The preparation of the recombinant Translin was carried out on a thin carbon film applied to a mesh copper grid and negatively stained with potassium phosphotungstate.(Kasai *et al.*, 1997).

B. Octameric Translin picture (a) magnified-scale, bar is 1000Å and (b) electron microscope structure reconstructions of Translin.
Adapted from (VanLoock *et al.*, 2001).

1.7.1 Translin role in mitosis

During the course of studies comparing basal expression levels of various proteins during mitotic cell division, it was shown that there is link between the rate of cell proliferation and Translin levels (Ishida *et al.*, 2002).

Translin expression was found to be periodic during the cell cycle; Translin synthesis starting in S phase and becoming maximal during the G2/M phase, (Ishida *et al.*, 2002). Maximal expression during S and mitosis phases suggesting functions in the replication of chromosomal DNA or cell division control (Ishida *et al.*, 2002).

Translin localization was analysed by confocal microscopy (Figure 1.5) which revealed a dispersed distribution of Translin in the cytosol. At prophase Translin was detected on spindle poles (centrosomes) which have also been observed in *Xenopus* oocytes (Reviewed in Ishida *et al.*, 2002). When the cells entered mitosis, Translin was seen on the astral microtubules, which radiate in all directions from the centrosomes at prometaphase/metaphase. This suggests a significant function of Translin in cell division can be attributing to acceleration of microtubule organization and chromosome separation through mitosis (Ishida *et al.*, 2002).

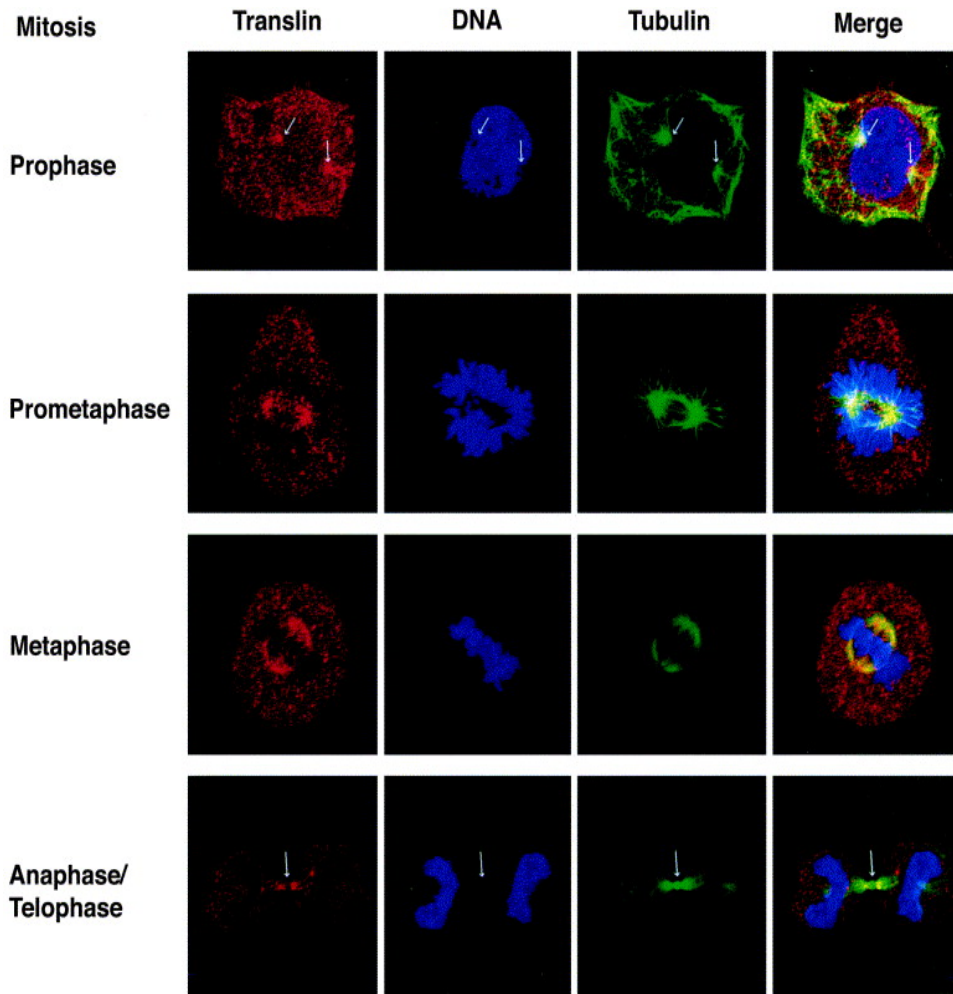


Figure 1.5 Translin localization to the centrosomes.

Translin localizes to the centrosomes bipolar mitotic spindles and midzone. HeLa cells at prophase, prometaphase, metaphase and anaphase/telophase were stained Hoechst 33258 for the nucleus (blue), FITC anti- α -tubulin (green), and anti-Translin followed by goat anti-rabbit IgG-TRITC (red). Localization of tubulin and Translin was examined by confocal laser scanning microscopy as described previously (Figure from Ishida *et al.*, 2002).

1.7.2 Translin and chromosomal breakpoints

Some studies have suggested that Translin and Trax form a complex (for example, Ye *et al.*, 2011; Tian *et al.*, 2011). This complex has been proposed to be involved in various biological processes, such as RNA metabolism, the regulation of genome stability, and carcinogenesis. The exact biological function of the complex remained to be fully elucidated (Tian *et al.*, 2011) although the finding of a role in tRNA processing is a significant step forward (Li *et al.*, 2012). As mentioned in the previous section, human Translin has a higher and more specific affinity for binding consensus sequences at the breakpoint junction of tumours (Chalk *et al.*, 1997; Hosaka *et al.*, 2000). In a study by Jacob *et al.* (2004), it was reported that human Translin had strong affinities for the single-stranded overhangs of microsatellite repeats [d(GT)_n] and G-strand telomeric repeats (d(TTAGGG)_n] than for lymphoid cancer-associated translocation Bcl-CL1 consensus sequences. The microsatellite repeats of d(GT)_n and d(AC)_n are related to recombined hotspots (Majewski and Ott, 2000). Therefore, Translin may play a role in recombination at microsatellites, where single-stranded d(GT)_n microsatellite overhangs are the intermediates of recombination. These microsatellite repeats might be related to the susceptibility genes for several human diseases, such as allergies, schizophrenia, and colon cancer (Tamura *et al.*, 2001; Itokawa *et al.*, 2003; Komarova *et al.*, 2002; Hirai *et al.*, 2003). So far, it is not clear if a mutation in the Translin gene has a relationship with carcinogenesis. The specific chromosome rearrangement t(12;16)(q13;p11) creates genetic fusion of the TLS and CHOP genes in myxoid liposarcomas, resulting in the transcriptional abnormality of these two genes (Wie *et al.*, 2003). Translin recognition sites were found in 10 out of 11 liposarcomas, with TLS-CHOP fusion genes being investigated (Hosaka *et al.*, 2000).

1.8 Chromatin

Within a eukaryotic cell the genetic material is organised in long DNA molecules which are associated with a number of highly conserved proteins; these macro molecules are modified in a variety of ways to form an organised and dynamic structure called chromatin. This complex supports and controls crucial genome functions (Fischle *et al.*, 2003; Ehrenhofer-Murray, 2004). The fundamental component of chromatin is a repeating unit nucleosome comprised of 147 base pairs of DNA which are on the outer surface of a core of eight proteins (Goto and Nakayama, 2012). This complex is comprised of two copies each of the core histone proteins H2A, H2B, H3 and H4. The nucleosomes are linked to each other for the purposes of stabilizing the complex of nucleoproteins by linker DNA which is bound by histone H1 (Szerlong and Hansen, 2011; Olsson and Bjerling, 2011; Jansen and Verstrepen, 2011). Silencing or expression of genes is dependent on the chromatin state whereby, histones have a charged NH₂- terminus, known as the histone tail, which is targeted by enzymes responsible for chromatin modification (Jenuwein and Allis, 2001). The histone tails are linked to the regulation of chromatin by post translational Modified such as methylation of H3 residues (K4, K9, K27, K36 and K79) and K20 of H4, this process of methylation can either mark chromatin as transcriptionally active or inactive, dependent on the modified lysine residue (Martin and Zhang, 2005). Chromatin packaging and accurate regulation are thus very important. The arrangement of different states of chromatin in distinct temporal patterns requires the involvement of a large number of factors and processes. Depending on the cell conditions chromatin can be susceptible to different factors for distinct genetic processes (Olsson and Bjerling, 2011); For example, errors of oncogene and tumour suppressor gene expression resulting from the lack of regulation of chromatin lead to cancer.

There are two types of chromatin (Fig 1.6). Transcriptionally active chromatin is called euchromatin, which is less-condensed and more open enabling gene expression; it is generally associated with acetylation of histones H3 and H4. In contrast, the second type, heterochromatin, is characterised by hypoacetylation and methylation of histone H3 at H3K9, which is considered to be a key modification required for heterochromatin formation by binding to heterochromatin protein 1 (HP1) (Olsson and Bjerling, 2011; Gerace *et al.*, 2010).

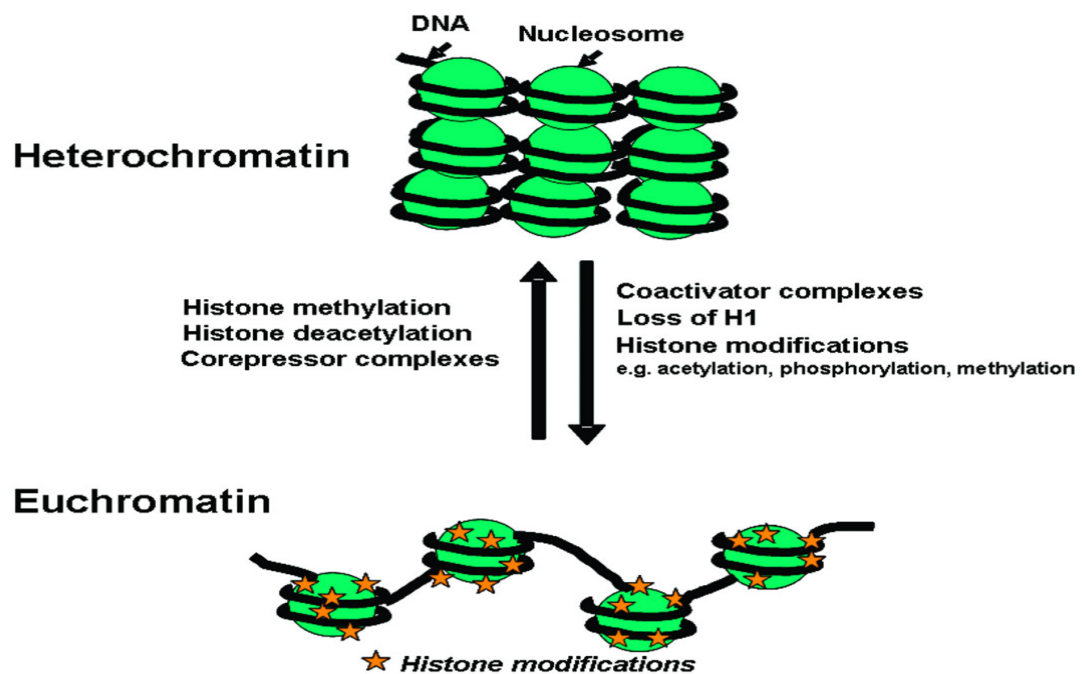


Figure 1.6 Heterochromatin and Euchromatin.

Heterochromatin is the firmly packed "closed" form of chromatin, associated with gene repression through a mechanism like histone methylation, acetylation or siRNA during RNAi. Activation of chromatin by enzymes which can acetylate, phosphorylate or methylate histone tails, lead to more "open" form which allows genes to be expressed (Adcock *et al.*, 2006).

Heterochromatin is highly condensed, and is transcriptionally inactive. The opening of chromatin in euchromatin regions occurs through a specific co-activator complex which has enzymes that have the ability to effect acetylation, phosphorylation, or methylation of histone tails. In addition to its regulating roles, chromatin is implicated in the response of the cellular to DNA damage, particularly DSBs (Peng and Karpen, 2009).

1.8.1 Euchromatin and heterochromatin functions.

The formation and mechanism of heterochromatin appears to be conserved from yeast to mammals (Goto and Nakayama, 2012). Heterochromatin has at least two roles. The first role is to silence genes in repetitive DNA sequences (Fukagawa *et al.*, 2004). The second role is necessary for the normal function of specific genomic regions such as centromeres, telomeres and rDNA, which in the majority of eukaryotes, are heterochromatic in nature. The sequence of centromeric and telomeric DNA is generally not conserved but comprised of repetitive sequences, pieces of transposons, and very few genes (Djupedal and Ekwall, 2009). Centromeres are embedded in heterochromatin which is necessary for their normal function, and so it is required for the segregation of chromosomes. Heterochromatin is also required for the protection of telomeric integrity at the end of the chromosome (Djupedal and Ekwall 2009; Fukagawa *et al.*, 2004; Goto and Nakayama, 2012). In addition, heterochromatin plays a crucial function in protecting the genome from DNA elements such as transposons, which are repressed by heterochromatin (Djupedal and Ekwall 2009). In *S. pombe*, the centromere consist of inverted repetitive sequences which are necessary to generate small interfering RNAs (which will be discussed in greater detail later on), which make a specific guide for targeting heterochromatin. Furthermore, reporter genes that are placed inside or near the heterochromatin sites are generally associated with gene

expression silencing (Cam *et al.*, 2005; Grewal and Jia, 2008; Yasuhara and Wakimoto, 2006).

In several eukaryotes, including *S. pombe*, heterochromatin formation is promoted by hypoacetylation and hypermethylation of histone H3 at lysine 9 (Grewal and Elgin, 2002). Mutations in proteins that regulate heterochromatin result in defects in heterochromatin assembly and this can cause dysfunction of genome stability regulation (Grewal and Jia, 2008; Shimada and Murakami, 2010).

1.9 Centromeres

Centromeres are specialised regions of chromosomes on which the kinetochore, a complex of nucleoproteins, is assembled. The centromeres ensure the regulation and maintenance of sister chromatin attachment, and accurate and proper segregation of replicated genetic material through the processes of mitosis and meiosis (Ekwall *et al.*, 1999). Thus the sister kinetochores form the attachment sites for capturing the spindle microtubules and directing chromosomal movement to the spindle poles (Shiroiwa *et al.*, 2011). Therefore, any defect in the role of centromeres leads to mis-segregation by loss or gain of the chromosomes, and that is implicated in genetic diseases and tumours (Ekwall *et al.*, 1999). The three centromeres of *S. pombe* are large and complex (Fig. 1.7). They span approximately 35-110 kb (Shiroiwa *et al.*, 2011), and are comprised of central parts of unique non-repetitive DNA (*cnt*) sequences that are surrounded by two inverted inner repeats most (*imr*) which share identical sequences within a centromere, but differ between the three centromere. These are additionally flanked by the outer repeats (*otr*) (Fig. 1.7) (Goto and Nakayama, 2012; Smirnova and McFarlane, 2002). Inserting a gene within any region of the *S. pombe* centromeres leads to transcriptional repression. The centromeres of *S. pombe* have

considerable similarities to those of high eukaryotic cells, and *S. pombe* provides an excellent model system for studying the function of centromeres (Ekwall *et al.*, 1999).

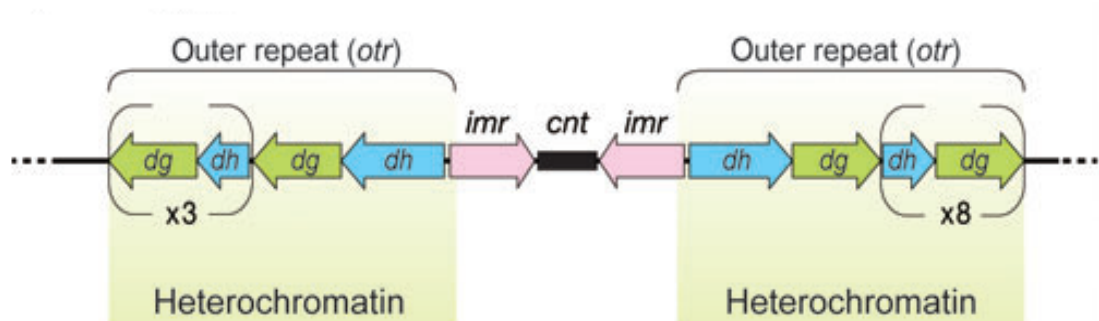


Figure 1.7 The centromere regions of the three *S. pombe* chromosomes are arranged in a similar way.

Every centromere comprised of a central core (*cnt*) which is surrounded inner most repeats (*imrs*) which in turn are surrounded by outer inverted repeat regions (*otr*). The *otrs* have multiple copies of *dg* and *dh* repeat sequences. The centromeres differ in the number of repeated sequences (Goto and Nakayama, 2012). It is from these *otrs* that the majority of transcription of centromeres are initiated (Martienssen, 2011).

1.9.1 Role of the *tRNA* genes as a chromatin barrier

The centromere region of the chromosomes of eukaryotic organisms is heterochromatic in form and plays an important role in faithful segregation (Grewal and Elgin, 2002; Takahashi *et al.*, 1991; Kuhn *et al.*, 1991). The heterochromatin in *S. pombe* and several other eukaryotes cells is created by hypoacetylation and hypermethylation of histone H3 at lysine 9 (Grewal and Elgin, 2002). The spreading of heterochromatin is restricted by moderately repetitive DNA sequences known as chromatin insulators (Raab *et al.*, 2011; Sun and Elgin, 1999). Chromatin barriers prevent the spread of pericentromeric heterochromatin from invading active domains; therefore, the absence of this barrier leads to defects in chromosome separation, indicating that it is needed by the centromere so it can play an accurate role (McFarlane and Whitehall, 2009; Scott *et al.*, 2007). The centromere regions of the fission yeast *S. pombe* have a high-density distribution of *tRNA* genes, either individually or in tiny clusters, which are located between the heterochromatin and euchromatin domains where they block the extension of heterochromatin in yeast (White, 2011; Iwasaki *et al.*, 2010; Halder and Kamakaka, 2006). The *tRNA* gene is transcribed by the transcription factors TFIIB and TFIIC along with RNA pol III (RNA polymerase III is essential for the transcription of eukaryotic *tRNA* genes) (Raab *et al.*, 2011; Takahashi *et al.*, 1991; Kuhn *et al.*, 1991). The transcriptional potential of the *tRNA* genes is vital for the insulator role in which the mutation in *tRNA* promoter sequences is critical for barrier function; mutations in the *tRNA* promoter elements or in the factors that bind the promoter eliminate or diminish insulator activity (Scott *et al.*, 2007; Oki and Kamakaka, 2005). *cen1* in *S. pombe* relies on an undamaged *tRNA^{Ala}* gene (*cen1 tRNA^{Ala}*), where it blocks the extension of heterochromatin and allows the *ura4⁺* reporter gene expression, which is situated near the centromere (Scott *et al.*, 2006).

1.10 RNAi

RNA is one of the building blocks of life; it is not just important for translation of genetic information to proteins, but it regulates expression of genes (Meister and Tuschl, 2004). A wide variety of eukaryotic organisms have pathways that implicate RNA as a trigger to regulate numerous cellular processes. For example, small interfering RNAs (siRNAs), which are a type of double-stranded RNA vary in length between 21 and 30 nucleotides, plays an important role in gene expression and/or modification of chromatin (Bayne *et al.*, 2010). This generates transcriptionally inactive chromatin, including the formation of heterochromatin (Bühler and Moazed, 2006). siRNAs also regulate gene expression by a variety of routes such as targeting mRNA degradation, inhibition of translation, and methylation of histones (Czech and Hannon, 2010), it also protects a cell from invading RNA viruses, and represses selfish genomic sequences such as transposons (Kawamata and Tomari, 2010; Meister and Tuschl, 2004; Carthew and Sontheimer, 2009). In addition, in *S. pombe* mating-type loci and telomeric sequences with homology to centromeric outer repeats use components of the siRNA pathway for generation heterochromatin (Bayne *et al.*, 2010). Centromeric siRNAs are either generated internally during S phase where RNA polymerase II in *S. pombe* transcribes the outer repeat elements from both DNA strands to double-strand RNA (dsRNA) (Djupedal and Ekwall 2009; Lejeune and Allshire, 2011), or for other regions they are exogenous (e.g., from viral replication) (Wolfswinkel and Ketting, 2010; Bayne, *et al.*, 2010). These dsRNAs are simultaneously processed to siRNAs of 21-30 nucleotides through slicing by Dicer1, a member of the RNaseIII family of ribonucleases (Colmenares *et al.*, 2007; Bühler and Moazed, 2006; Grewal and Jia 2007; Matzke and Birchler, 2005; Reyes-Turcu and Grewal, 2012). Then these siRNAs, which are cognate to repeat sequences located at places of heterochromatin formation, are loaded onto a silencing effector complex such as RISC (RNA-induced silencing complex)

(Matzke and Birchler, 2005). RISC contains some effector proteins like argonaute, which has the ability to slice endogenous message by its endonuclease domain and is responsible for guiding siRNA to cognate sequences for inactivation. After the loading of siRNA duplexes into RISC and successful processing annealing of siRNA to its target RNAs, the double-strand of siRNA is separated, so one strand-called the passenger strand, will be discarded and the other strand will be retained to guide the RISC complex to its target mRNA (Kawamata and Tomari, 2010; Volpe and Martienssen, 2011; Malone and Hannon, 2009). A Trax and Translin heterodimer known as C3PO has been implicated in removing the passenger strand (Liu *et al.*, 2009) in *Drosophila*. In addition, siRNAs act like primers for an enzyme known as an RNA-dependent RNA polymerase (RdRP), which is implicated in the synthesis of new small interfering RNAs (siRNA) in some organisms by using the targeted complementary RNA to generate further dsRNA sequences (Figure 1.8) (Volpe and Martienssen, 2011). Chromatin can therefore be targeted by base-pairing in RISC through the interaction of siRNA with H3K9 methylated nucleosomes (Verdel *et al.*, 2004), which leads to repression of the homologous gene either by post-transcriptional or transcriptional-based mechanisms (Lejeune and Allshire, 2011). This leads to the recruitment of an enzyme called Clr4, which is a histone lysine methyltransferase that is required for histone H3K9 methylation (Lejeune and Allshire, 2011; Reyes-Turcu and Grewal, 2012). The capability of Clr4 to modify the H3K9 by methylation is pivotal for distribution of heterochromatin (Reyes-Turcu and Grewal, 2012). Subsequently, the modified form of H3-K9 recruits a chromodomain protein called Swi6 (the fission-yeast HP1 homologue) to bind to the methylated lysines, which reduces transcriptionally ability and initiates distribution of the silent chromatin (Malone and Hannon, 2009; Martin and Zhang, 2005) (Figure 1.8).

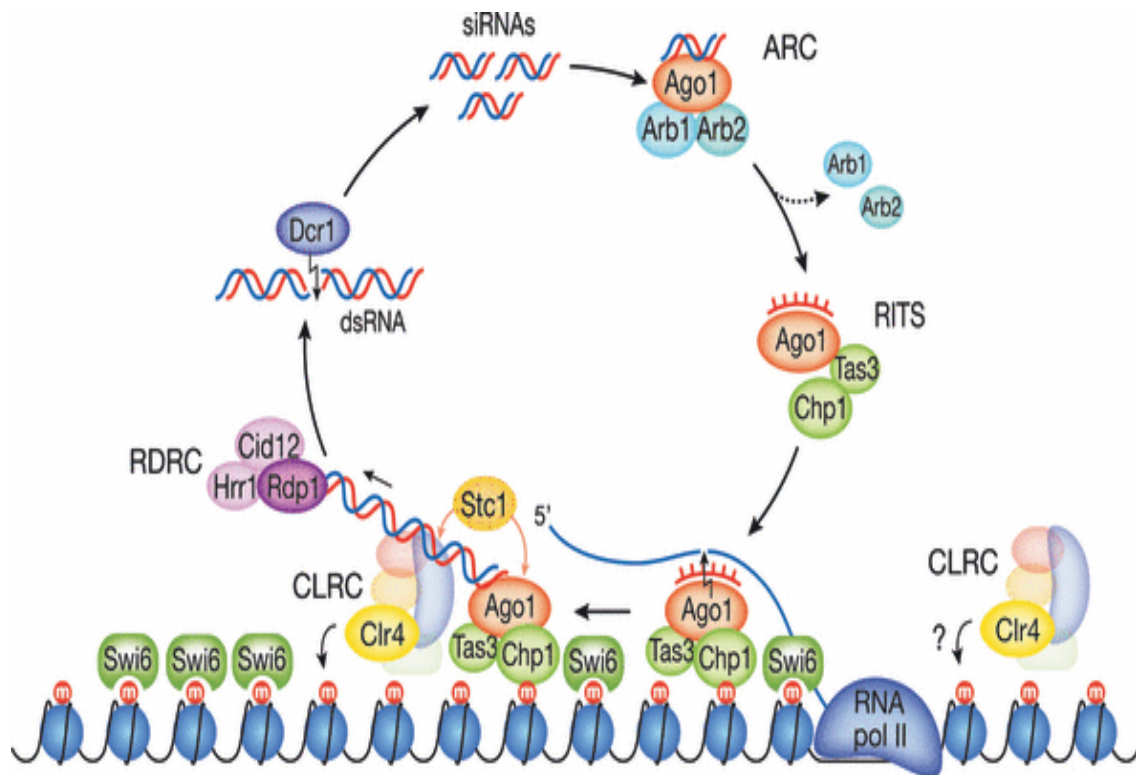


Figure 1.8 The epigenetic silencing mechanism in *S. pombe*.

Heterochromatin DNA (black line) surrounded nucleosomes (blue circle). Histone H3 lysine 9 methylation (H3K9me; red circles “m”) is methylated. The activated RNAi-induced transcriptional silencing RITS complex target the nascent transcript which is produced by RNA polymerase II. RITS interacts with H3K9me by the Chp1 subunit. New dsRNA from the transcripts is synthesis by RDRC. Then Dcr1 slices it to siRNAs. This siRNAs are cleaved into single-stranded siRNAs and load again into RITS complex. Then the Clr4 is recruited for dispersal of gene silencing into nearby regions. (Adapted from Goto and Nakayama, 2012).

1.11 Swi6 function in heterochromatin

The chromatin status is related to the hyperacetylation of histones, in particular to the Lys-9, Lys-14 and Lys-4 of histone H3. In contrast, the hypoacetylation of H3 at Lys-9 and Lys-14 by histone deacetylases Clr3, Clr6 and Sir2, results in H3-Lys-9 methylation by the histone methyltransferase Clr4 (Haldar *et al.*, 2011). Methylation of H3-Lys-9 is initiated by RITS (see Section 1.10 for further details). Where, H3K9me recruits the heterochromatin protein Swi6 (HP1), which results in the suppressed transcription of the gene in the pericentromeric heterochromatin. More Clr4 methylase is recruited by Swi6 for histone methylation extension and additional Swi6 (HP1) binding until it reaches boundary regions (Li *et al.*, 2011; Chinen *et al.*, 2010). Swi6 has an important function in the function of the centromeres and correct chromosome separation, so deletion of Swi6 in cells leads to missegregation of chromosomes during anaphase and a higher rate of chromosome loss (Keller *et al.*, 2012; Hayashi *et al.*, 2009). Interestingly, in addition to its function in facilitating accuracy in the centromeric function, Keller *et al.* (2012) in their recent study of *S. pombe*, demonstrated that Swi6 suppresses heterochromatic genes by removing captured heterochromatic transcripts and guiding heterochromatic mRNAs to final degradation (Figure 1.9).

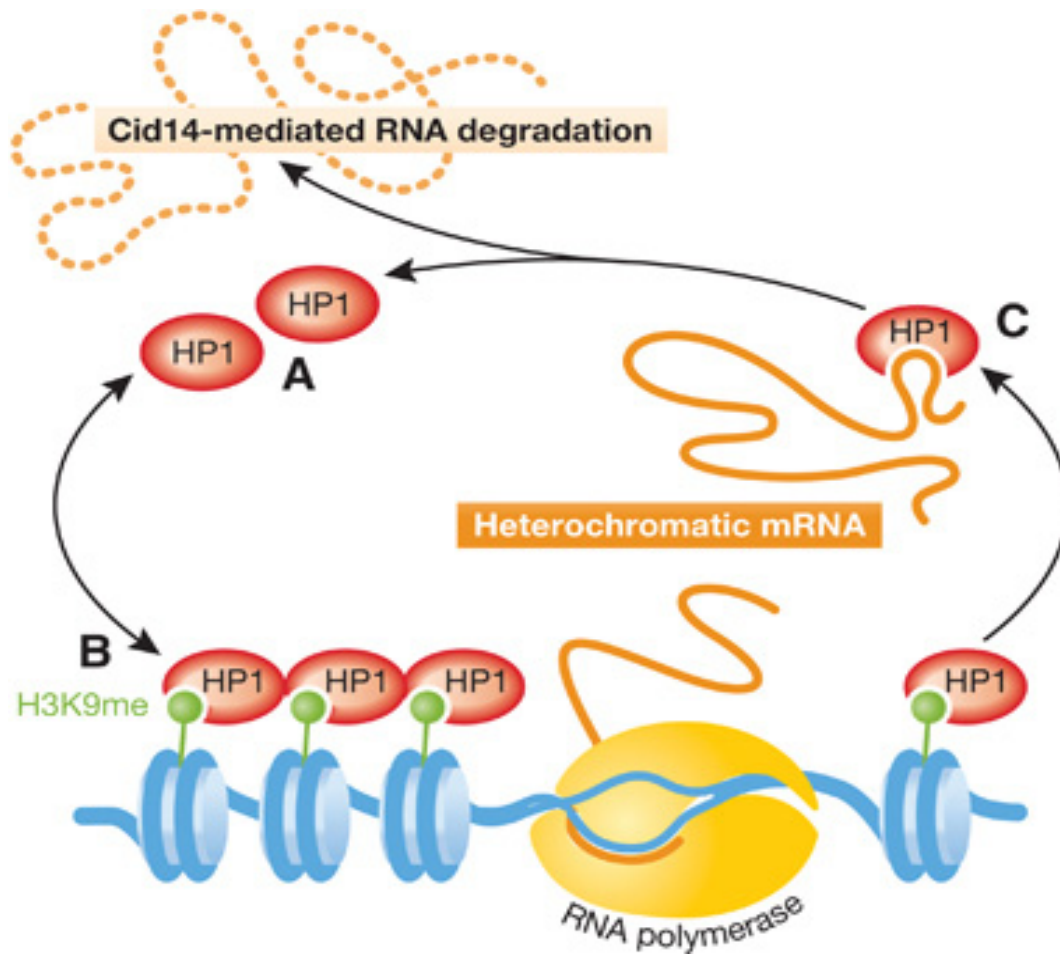


Figure 1.9

The image shows the changed state of $HP1^{Swi6}$ between free $HP1^{Swi6}$ (A) and H3K9me-bound (B). (C) Heterochromatin is transcribed by RNA polymerase; however, the heterochromatic transcripts are captured by $HP1^{Swi6}$ and taken to Cid14 (Poly [A] RNA polymerase), which is needed in polyadenylation for the destruction of the heterochromatin RNA transcript (adapted from Ren *et al.*, 2012).

1.11 *S. pombe* as a model eukaryote

S. pombe is an African brewing yeast, which was originally isolated from millet beer and named by Lindner and then developed as an experimental model by Urs Leupold in the 1950s (Nurse, 2002). In 2002, the genome of *S. pombe*, which contains around 5000 genes was fully sequenced (Wood *et al.*, 2002). The genome size is approximately 13.8 Mb, distributed among just three chromosomes, 3.5, 4.6 and 5.7 Mb in size (Wood *et al.*, 2002). There are many reasons why *S. pombe* is a popular model organism for researchers. The primary reason is that it is facile and genetically tractable. It also exists in the haploid state in normal growth conditions and gene manipulation techniques are widely used (for example, see Bahler *et al.*, 1998). In addition, it has a relatively short doubling time of only two to four hours. *S. pombe* has also recently become an excellent tool for the studying of the epigenetic and RNAi. *S. pombe* has a single copy of every one of the major genes of RNAi, which is crucial for transcriptionally repressed chromatin assembly and regulation sited at centromeres, mating type loci and telomeres these genes are not found in *Saccharomyces cerevisiae* (Martienssen *et al.*, 2005). We aimed in this study if Translin has redundat pathway with Dicer or Argonaute and their function in centromeric heterochromatin, and genom stability, and investigate any role for the double mutants of Translin and Dicer or Translin and argonaute in DNA damage.

Chapter 2

Materials and Methods

2.1 Yeast meiotic crosses

Samples of 2.5×10^7 *S. pombe* cells of opposite mating type (h^+ and h^-) were cultured in standard full media in 5 ml yeast-extract liquid (YEL), which was supplemented with 100 $\mu\text{g/ml}$ adenine, uracil, leucine, and then mixed in a 1.5 ml Eppendorf microcentrifuge tube in equal volumes of approximately 750 μl for each strain. The cells were subjected to microcentrifugation and the supernatant was aspirated. The pellet was rewashed with 1 ml of sterile distilled water then spun down again; then all the pellets were resuspended in 50 μl of distilled sterile water then spotted on SPA and incubated for 3-4 days. Asci and unmated cells were scrapped from each spot into 1 ml of 0.6% β -glucuronidase (Sigma) solution in an Eppendorf tube. The suspension was mixed and incubated for 16 hours at 25°C to release spores from the asci and to kill vegetative cells, then ethanol was added to 30% and the suspension was incubated at room temperature for not more than 5 minutes. The spores were spun down for 1 min in an Eppendorf microcentrifuge, the supernatant was removed and the cell pellets were resuspended in 1 ml of distilled sterile water. Dilutions were plated on the suitable media (YEA) to allow growth of viable spores. Plates were incubated for 3 days at 33°C , and colonies were replica plated onto selective media as required.

2.2 Iodine staining for mating-type test

S. pombe cells of the opposite mating type (h^+ and h^-) that produce spores have a starchlike compound not found in vegetative cells when grow in fully supplemented sporulation media (SPA) after incubation for 3 days, after that the plates exposure to iodine so

materials that have spores change to black (positive iodine reaction) after a 5 minute exposure to iodine vapour. Material containing only vegetative cells turn yellow (negative iodine reaction).

2.3 Cell lysis and *S. pombe* genomic DNA extraction

A single colony was picked and inoculated into 5 mls of yeast extract liquid (YEL) and incubated overnight (16 hours) or until saturated in shaking incubator. The temperature was optimised at 33°C. The cells were collected at 4,000 r.p.m. the pellets were washed with 1 ml of distilled sterile H₂O, then the pellet was resuspended in 200 µl of lysis buffer and 0.3 g of sterile acid washed beads were added. Then 100 µl of phenol-chloroform (1:1) were added. This was then vortexed 3 times for 30 seconds, briefly incubating on ice between each vortex, and then spun down for 12 minutes at 13,000 r.p.m. The upper layer was then removed and DNA was precipitated by adding 1 ml 100% ethanol, and then the ethanol was aspirated off and the pellet was air dried (10-15 minutes). The pellet was washed once using a solution of 70% ethanol. The pellet was then resuspended 100 µl TE (10 mM Tris-HCL [pH 7.5] and 1 mM EDTA [pH 8.0]).

2.4 DNA purification using phenol chloroform

An equal volume (1:1) of PC (phenol/chloroform) with 0.1 M NaCl was added to the DNA solution, mixed well, and spun in a microfuge at 13,000 r.p.m. at room temperature for 15 min. The top (aqueous) layer that contained the DNA was moved to new a Eppendorf tube. Three volumes of cold ethanol 100% was added. This was gently mixed and put in at -20°C overnight or in -80°C for 1 hour for DNA precipitation, and then the DNA was microfuged at 13,000 r.p.m. for 15 minutes. The DNA pellet was washed carefully at room temperature in 1 ml 70% ethanol and spun down at 13,000 r.p.m. in microcentrifuge. The supernatant was removed and then the pellet was air dried at room temperature. The pellet was then dissolved in TE buffer pH 8.0.

2.5 Media recipes

The media used in this project was mostly obtained from Difco (Becton Dickinson) and Sigma. Extracts used were full media yeast extract (YE), synthetic Edinburgh minimal medium 2 (EMM2), and the sporulation media malt extract (ME). The appropriate nucleotide amino acid and supplement were added to a final concentration of 200 mg/l.

Table 1 Table 2.1 Media recipes

1. YEA	1 LITRE
Yeast Extract	5 g
Glucose	30 g
Agar	14 g
2. YEA with Geneticin	
YEA	500 ml
Geneticin (50 mg/ml stock solution)	1 ml
500 mg geneticin was dissolved in 10 ml sterile distilled water for stock solution. The YEA+ Geneticin media was wrapped in aluminium foil and stored at 4°C.	
3. SPA	500 ml
Glucose	5 g
KH ₂ PO ₄	0.5 g
Agar	15 g
*Vitamins (x1000)	0.5 ml
1 ml 1000 x Vitamins in every 500 ml *Added after autoclaving media	

4. EMM2	1 LITRE
Potassium Hydrogen Phalate	3 g
Na ₂ HP0 ₄	2.2 g
NH ₄ Cl	5 g
Glucose	20 g
*Vitamins (x1000)	1 ml
*Minerals (x 10,000)	0.1 ml
*Salts (x50)	20 ml
Agar	14 g
*To add after autoclaving media	
5. SALTS x 50	500 ml
MgCl ₂ .6H ₂ O	26.25 mg
CaCl ₂ .2H ₂ O	0.3675 g
KCl	25 g
Na ₂ SO ₄	1 g
6. VITAMINE X 1000	500 ml
Pantothenic acid	0.5 g
Nicotinic acid	5 g
<i>Myo</i> -inositol	5 g
Biotin	5 mg
7. MINERALS X 10,000	500 ml
Boric acid	2.5 g
MnSO ₄ .7H ₂ O	2 g
FeCl ₂ .6H ₂ O	1 g

KI	0.5 g
Molybdic acid	0.2 g
CuSO ₄	0.2 g
Citric acid	5 g
*After autoclaving add a few drops of 1:1:2 chlorobenzene/ dichloroethane/ chlorobutane	
8. NITROGEN BASE AGAR (NBA)	1 LITRE
Nitrogen base	1.7 g
Glucose	10 g
(NH ₄) ₂ SO ₄	5 g
Agar	24 g
9. 100 X DENHARDT'S SOLUTION	50 ml
Polyvinylpyrrolidone (MW40,000)	1 g
Bovine Serum Albumin	1 g
Ficoll 400	1 g
10. 20 X SSC	1 LITRE
3 M NaCl	175.35 g
0.3 M Sodium Citrate Dihydrate	88.23 g
11. 20 X SSPE	1 LITRE
3M NaCl	175.3 g
0.2 M NaH ₂ PO ₄ -H ₂ O 27	6 g

0.02 M EDTA-Na (0.5 M stock solution)	40 ml
Distilled water	800 ml
Adjust pH to 7.4 with NaOH	
Add distilled water to make 1 litre	
12. LB Broth.	1 LITRE
Bacto-tryptone	10 g
Yeast extract	5 g
Na Cl	10 g
13. Drugs	
5-FOA (300 and 400ug/ml)	(300 and 400ug/ml)
TBZ (15ug/ml)	(15ug/ml)
MMS (0.0025, 0.005, 0.0075%)	(0.0025, 0.005, 0.0075%)
Mitomycin C (0.15mM)	(0.15mM)
Phleomycin (1, 2.5, 5, 10ug/ml)	(1, 2.5, 5, 10ug/ml)
Hydroxyl urea (3, 5, 10mM)	(3, 5, 10mM)

Table 2.1 The *S. pombe* strains used in this study.

Strain Number	Genotype	Source
BP1	<i>h⁻ 978</i> (Wild type)	McFarlane collection
BP8	<i>h⁺ 975</i> (Wild type)	McFarlane collection
BP90	<i>h⁻ ade6-M26 ura4-D18 leu1-32</i>	McFarlane collection
BP91	<i>h⁺ ade6-52 ura4-D18 leu1-32</i>	McFarlane collection
BP153	<i>h⁻ mis6-302</i> (incubate at 25 °C)	McFarlane collection
BP187	<i>h⁻ mis4-242</i> (incubate at 25 °C)	McFarlane collection
BP846	<i>h⁺ ade6-M210 hop1::kanMX6</i>	McFarlane collection
BP1079	<i>h⁻ ade6-M26 ura4 -D18 leu1-32 Tsn1::kanMX6</i>	McFarlane collection
BP1080	<i>h⁻ ade6- M26 ura4-D18 leu1-32 Tsn1::kanMX6</i>	McFarlane collection
BP1089	<i>h⁻ ade6-M26 ura4 -D18 leu1-32 trx::kanMX6</i>	McFarlane collection

BP1090	<i>h⁻ ade6-M26 ura4 -D18 leu1-32 trax::kanMX6</i>	McFarlane collection
BP1162	<i>h⁺ bub1::kan^R ura4-D18 leu1-32</i>	McFarlane collection
BP1731	<i>h⁺ ade6- M26 rec10-175 ::kanMX6</i>	McFarlane collection

BP2203	<i>h⁻ cntTM1(Nco1)::ura4 ura4-DS/E leu1-32 ade6- M 210</i>	McFarlane collection
BP2204	<i>h⁻ otrR1(Sph1)::ura4 ura4-DS/E leu1-32 ade6- M 210</i>	McFarlane collection
BP2221	<i>h⁻ ago1::kanMX6 ade6- M 210 ura4-DS/E leu1-32</i>	McFarlane collection
BP2294	<i>h⁻ ade6- M 210 leu1-32 ura4-D18 Ch16-23R</i>	McFarlane collection
BP2385	<i>h⁻ cntTM1(Nco1)::ura4 ura4-DS/E leu1-32 ade6- M 210 ago1::kanMX6</i>	McFarlane collection
BP2386	<i>h⁻ imr1(Nco1)::ura4 ori1 ura4-DS/E leu1-32 ade6- M 210 ago1::kanMX6</i>	McFarlane collection
BP2388	<i>h⁻ otrR1(Sph1)::ura4 ura4-DS/E leu1-32 ade6- M 210 ago1::kanMX6</i>	McFarlane collection
BP2406	<i>h⁻ ade6- M 210 leu1-32 ura4-D18 Ch16-23R trax::kanMX6</i>	This study
BP2413	<i>h⁻ otrR1(Sph1)::ura4 ura4-DS/E leu1-32 ade6-m210 trax::kanMX6</i>	This study
BP2414	<i>h⁹⁰ ade6-M26 leu1-32 ura4-D18 tsn1::kanMX6</i>	This study
BP2417	<i>h⁺ ade6-52 ura4-D18 leu1-32 tsn1::kanMX6</i>	This study
BP2418	<i>h⁻ cntTM1(Nco1)::ura4 ura4-DS/E leu1-32 ade6- M 210 tsn1::kanMX6</i>	This study
BP2420	<i>h⁻ otrR1(Sph1)::ura4⁺ ura4-DS/E leu1-32 ade6- M210 tsn1::kanMX6</i>	This study
BP2421	<i>h⁻ ade6-M210 leu1-32 ura4-D18 Ch16-23R tsn1::kanMx6</i>	This study

BP2472	<i>h⁻ ade6-M210 leu1-32 ura4-D18 Ch16-23R ago1::kanMx6</i>	McFarlane collection
BP2501	<i>h⁻ imr1(Nco1)::ura4⁺ ori1 ura4-DS/E leu1-32 ade6-M210 tsn1::KanMX6</i>	This study
BP2506	<i>h⁻ imr1(Nco1)::ura4⁺ ori1 ura4-DS/E leu1-32 ade6-M210</i>	This study
BP 2704	<i>h⁺ rDNA::ura4⁺ ura4-DS/E ade6-M210</i>	McFarlane collection
BP2705	<i>h⁺ Ch16-M23::ura4⁺ TEL[72] ura4-DS/E leu1-32 ade6-M210 (Ch16 ade6-M216)</i>	McFarlane collection
BP2706	<i>h⁹⁰ mat3-M (EcoRV)::ura4⁺ ura4-DS/E leu1-32 ade6-M210</i>	McFarlane collection
BP2720	<i>h⁺ rDNA::ura4⁺ ura4-DS/E ade6-M210 tsn1::kanMX6</i>	This study
BP2721	<i>h⁺ rDNA::ura4⁺ ura4-DS/E ade6-M210 tsn1::kanMX6</i>	This study
BP2722	<i>h⁺ Ch16-M23::ura4⁺ TEL[72] ura4-DS/E leu1-32 ade6-M210 (Ch16 ade6-M216) tsn1::kanMX6</i>	This study
BP2723	<i>h⁺ Ch16-M23::ura4⁺ TEL[72] ura4-DS/E leu1-32 ade6-M210 (Ch16 ade6-M216) tsn1::kanMX6</i>	This study
BP2724	<i>h⁹⁰ mat3-M (EcoRV)::ura4⁺ ura4-DS/E leu1-32 ade6-M210 tsn1::kanMX6</i>	This study
BP2725	<i>h⁹⁰ mat3-M (EcoRV)::ura4⁺ ura4-DS/E leu1-32 ade6-M210 tsn1::kanMX6</i>	This study
BP2747	<i>h⁻ ade6-M26 ura4-D18 leu1-32 dcr1::ura4</i>	This study
BP2748	<i>h⁻ ade6-M26 ura4-D18 leu1-32 tsn1::KanMX6</i>	This study

	<i>dcr1::ura4</i>	
BP2749	<i>h⁻ ade6-M26 ura4-D18 leu1-32 tsn1::KanMX6</i> <i>dcr1::ura4</i>	This study
BP2757	<i>h⁻ ade6-M26 ura4-D18 leu1-32 ago1::ura4</i>	This study
BP2758	<i>h⁻ ade6-M26 ura4-D18 leu1-32 ago1::ura4</i>	This study
BP2760	<i>h⁻ ade6-M26 ura4-D18 leu1-32 tsn1::KanMX6</i> <i>ago1::ura4</i>	This study
BP2811	<i>h⁻ ade6-M26 ura4-D18 leu1-32 rec10::KanMX6</i>	This study
BP2813	<i>h⁻ ade6-M26 ura4-D18 leu1-32 rec10::KanMX6</i>	This study
BP2894	<i>h⁻ ade6-M210 leu1-32 ura4-D18 Ch16-23R dcr1::</i> <i>ura4⁺</i>	This study
BP2895	<i>h⁻ ade6-M210 leu1-32 ura4-D18 Ch16-23R dcr1::</i> <i>ura4⁺</i>	This study
BP2899	<i>h⁻ ade6-M210 leu1-32 ura4-D18 Ch16-23R</i> <i>tsn1::KanMX6 dcr1:: ura4⁺</i>	This study
BP2900	<i>h⁻ ade6-M210 leu1-32 ura4-D18 Ch16-23R</i> <i>tsn1::KanMX6 dcr1:: ura4⁺</i>	This study
BP2973	<i>h⁻ ade6-M210 leu1-32 ura4-D18 Ch16-23R ago1::</i> <i>ura4⁺</i>	This study
BP2977	<i>h⁻ ade6-M210 leu1-32 ura4-D18 Ch16-23R</i> <i>tsn1::KanMX6 ago1::ura4⁺</i>	This study
BP2978	<i>h⁻ ade6-M210 leu1-32 ura4-D18 Ch16-23R</i> <i>tsn1::KanMX6 ago1::ura4⁺</i>	This study
BP2985	<i>h⁻ otrR1(Sph1)::ura4⁺ ura4-DS/E leu1-32 ade6-</i> <i>M210 dcr1::NatMX6</i>	This study
BP2989	<i>h⁻ cntTM1(Nco1)::ura4⁺ ura4-DS/E leu1-32 ade6-</i>	This study

	<i>M210 tsn1::kanMX6 dcr1::NatMX6</i>	
BP2995	<i>h- otrR1(Sph1)::ura4⁺ ura4-DS/E leu1-32 ade6-m210 tsn1::kanMX6 ago1::NatMX6</i>	This study
BP2996	<i>h- otrR1(Sph1):: ura4⁺ ura4-DS/E leu1-32 ade6-m210 tsn1::kanMX6 ago1::NatMX6</i>	This study
BP3001	<i>h⁻ cntTM1(Nco1):: ura4⁺ ura4-DS/E leu1-32 ade6-M210 ago1::NatMX6</i>	This study
BP3003	<i>h⁻ imr1(Nco1):: ura4⁺ ori1 ura4-DS/E leu1-32 ade6-M210 tsn1::KanMX6 ago1::NatMX6</i>	This study
BP3004	<i>h⁻ imr1(Nco1):: ura4⁺ ori1 ura4-DS/E leu1-32 ade6-m210 tsn1::KanMX6 ago1::NatMX6</i>	This study
BP3005	<i>h⁻ imr1(Nco1):: ura4⁺ ori1 ura4-DS/E leu1-32 ade6-M210 ago1::NatMX6</i>	This study
BP3024	<i>h⁻ otrR1(Sph1):: ura4⁺ ura4-DS/E leu1-32 ade6-M210 tsn1::kanMX6 dcr1:: NatMX6</i>	This study
BP3026	<i>h⁻ imr1(Nco1):: ura4⁺ ori1 ura4-DS/E leu1-32 ade6-M210 tsn1::KanMX6 dcr1:: NatMX6</i>	This study
BP3027	<i>h⁻ imr1(Nco1):: ura4⁺ ori1 ura4-DS/E leu1-32 ade6-M210 tsn1::KanMX6 dcr1:: NatMX6</i>	This study
BP3028	<i>h⁻ imr1(Nco1):: ura4⁺ ori1 ura4-DS/E leu1-32 ade6-M210 dcr1:: NatMX6</i>	This study
BP3029	<i>h⁻ imr1(Nco1):: ura4⁺ ori1 ura4-DS/E leu1-32 ade6-M210 dcr1:: NatMX6</i>	This study
BP3103	<i>h⁻ cntTM1(Nco1):: ura4⁺ ura4-DS/E leu1-32 ade6-M210 dcr1::NatMX6</i>	This study
BP3109	<i>h⁻ otrR1(Sph1):: ura4⁺ ura4-DS/E leu1-32 ade6-</i>	This study

	<i>M210 ago1::NatMX6</i>	
BP3111	<i>h⁻ cntTM1(Nco1)::ura4⁺ ura4-DS/E leu1-32 ade6-M210 tsn1::kanMX6 ago1::NatMX6</i>	This study
BP 2704	<i>h⁺ rDNA::ura4⁺ ura4-DS/E ade6-M210</i>	McFarlane collection
BP3154	<i>h⁺ rDNA::ura4⁺ ura4-DS/E ade6-M210 ago1::NatMX6</i>	This study
BP3213	<i>h⁺ rDNA::ura4⁺ ura4-DS/E ade6-M210 dcr1::NatMX6</i>	This study
BP2720	<i>h⁺ rDNA::ura4⁺ ura4-DS/E ade6-M210 tsn1::kanMX6</i>	This study
BP3193	<i>h⁺ rDNA::ura4⁺ ura4-DS/E ade6-M210 tsn1::kanMX6 ago::NatMX6</i>	This study
BP3189	<i>h⁺ rDNA::ura4⁺ ura4-DS/E ade6-M210 tsn1::kanMX6 dcr::NatMX6</i>	This study
BP3150	<i>h⁻ leu1-32 ura4-D18 T2R1-30292::ura4⁺</i>	McFarlane collection
BP3206	<i>h⁻ leu1-32 ura4-D18 T2R1-30292::ura4⁺ ago1::NatMX6</i>	This study
BP3153	<i>h⁻ leu1-32 ura4-D18 T2R1-30292::ura4⁺ dcr1::HYG</i>	This study
BP3214	<i>h⁻ leu1-32 ura4-D18 T2R1-30292::ura4⁺ tsn1::kanMX6</i>	This study
BP3220	<i>h⁻ leu1-32 ura4-D18 T2R1-30292::ura4⁺ ago1::NatMX6 tsn1::kanMX6</i>	This study
BP3236	<i>h⁻ leu1-32 ura4-D18 T2R1-30292::ura4⁺ dcr1::HYG tsn1::kanMX6</i>	This study
BP3151	<i>h⁻ leu1-32 ura4-D18 T2R1-7921::ura4⁺</i>	McFarlane collection

BP3208	<i>h⁻ leu1-32 ura4-D18 T2R1-7921:: ura4⁺</i> <i>ago1::NatMX6</i>	This study
BP3152	<i>h⁻ leu1-32 ura4-D18 T2R1-7921:: ura4⁺</i> <i>dcr1::HYG</i>	McFarlane collection
BP3216	<i>h⁻ leu1-32 ura4-D18 T2R1-7921:: ura4⁺</i> <i>tsn1::kanMX6</i>	This study
BP3238	<i>h⁻ leu1-32 ura4-D18 T2R1-7921:: ura4⁺</i> <i>ago1::NatMX6 tsn1::kanMX6</i>	This study
BP3218	<i>h⁻ leu1-32 ura4-D18 T2R1-7921:: ura4⁺</i> <i>dcr1::HYG tsn1::kanMX6</i>	This study
BP2706	<i>h⁹⁰ mat3-M (EcoRV)::ura4⁺ura4-DS/E leu1-32</i> <i>ade6-M210</i>	McFarlane collection
BP3155	<i>h⁹⁰ mat3-M (EcoRV)::ura4⁺ura4-DS/E leu1-32</i> <i>ade6-M210 ago1::NatMX6</i>	This study
BP3212	<i>h⁹⁰ mat3-M (EcoRV)::ura4⁺ura4-DS/E leu1-32</i> <i>ade6-M210 dcr1::NatMX6</i>	This study
BP2724	<i>h⁹⁰ mat3-M (EcoRV)::ura4⁺ura4-DS/E leu1-32</i> <i>ade6-M210 tsn1::kanMX6</i>	This study
BP3195	<i>h⁹⁰ mat3-M (EcoRV)::ura4⁺ura4-DS/E leu1-32</i> <i>ade6-m210 tsn1::kanMX6 ago::NatMX6</i>	This study
BP3190	<i>h⁹⁰ mat3-M (EcoRV)::ura4⁺ura4-DS/E leu1-32</i> <i>ade6-m210 tsn1::kanMX6 dcr::NatMX6</i>	This study

2.6 Methods of yeast transformation

2.7.1 Lithium Acetate (LiAc) yeast transformation

A single colony was inoculated in 50 ml of YEL or selective media overnight and incubated by shaking at 30°C to a density of $0.5-1 \times 10^7$ cells/ml. The cells were spun down at 3,000 r.p.m. for 5 min at room temperature in microcentrifuge. The medium was then poured off and the cells washed by gently adding 1 ml of distilled sterile water. The suspension was recentrifuged, and the pellet was transferred after resuspension to a new 1.5 ml microfuge tube and rotated again at 6000 r.p.m. for 1 min in microcentrifuge. The pellet was resuspended at 1×10^9 cells per ml in freshly made 0.1 lithium acetate (adjusted to pH 4.9 with acetic acid). 100 µl samples were transferred to a new Eppendorf tubes, with 2 µl of sheared herring testes DNA and 10–20 µg of transforming DNA (of a maximum volume of 10 µl). The suspensions were incubated at room temperature for 10 minutes and then 260 µl of 40% PEG/LiAc/TE was added and gently mixed. The mixture was incubated for 1 hour at room temperature. DMS was added after incubation and then the mixture was heat shocked at 42 °C for a minimum of 5 minutes. It was then left to cool at room temperature. The cells were harvested at 6,000 r.p.m. in a microcentrifuge after they were washed by adding 1 ml of distilled sterile water and spinning them down at 3,000 r.p.m. for 3 minutes. The supernatant was then discarded from the microfuge tube(s) and resuspended in 0.5 ml of distilled sterile water and 50 µl of the aliquots were plated out on YEA media plates and incubated for overnight, then make replica on selective media 100ug/l Nat antibiotics or onto EMM2 plates with or without required supplements for selection and incubated for up to 6 days at 30°C to test their ability to grow on nutrition selective media for Ura marker.

The cells will be selected in media contains 5-FOA, because orotidine-5'phosphate decarboxylase which has been encoded by *Ura4* for biosynthesis of uracil, convert FOA(5-fluoroorotic acid) to toxic compound 5-fluorouracil, so the cells that have *ura4* can be killed by selected media that contains FOA while *Ura-* cells are resistant to this compound.

2.7.2. Transformation of yeast by electroporation

Cells were grown in 50 ml YEL media until reaching a density of 1×10^7 cells per ml. The culture was then incubated in ice for 20 minutes. The sample was spun at 3,000 r.p.m. for 5 minutes at 4°C in a microcentrifuge the samples were washed 3 times in ice-cold 1 M Sorbitol before resuspending the sample in 1 M Sorbitol until reaching a density of 1×10^9 cells per ml. The electrocuvets (Geneflow) were pre-chilled on ice for 20 minutes, then 200 μl of the cell suspension was transferred into the electroporation chamber with 1 μg of transforming DNA dissolved in pure sterile distilled water (with a volume of $< 10 \mu\text{l}$), and the samples were shocked immediately at 2 kV. Then 1 ml of ice-cold 1 M Sorbitol was added.

2.8 Spot test

A single colony was grown and inoculated in 5 ml YEL and incubated overnight in a shaking's incubator until saturated. The temperature was optimised at 33°C . The cells were then transferred to a Eppendorf tube and spun down and resuspended in 1 ml of water at a concentration of approximately 5×10^6 cells /ml a serial dilution of 1:10 was made. Serial dilutions had 5 μl of each dilution spotted onto YES supplemented with appropriate drug as required. The plates were incubated for 3-4 days at appropriate temperature

2.9 *S. pombe* storage

For long term storage, one colony was grown in 5 ml YEL until saturation. Then 0.8 ml of YES was mixed with glycerol to a final concentration of 30%. Cultures were stored -80°C.

2.10 *E. coli* plasmid DNA extraction

Plasmid isolation methods were applied using a GenElute™ HP Plasmid Miniprep Kit. *E. coli* was grown overnight from the -70°C freezer stock of 5 ml of Luria Bertani (LB) media, which contained a suitable antibiotic. The culture was spun for harvest at 3,500 r.p.m. for 1 minute IN a microcentrifuge. The supernatant was isolated, and the bacteria pellet was left. The pellet was resuspended in 200 µl of kit resuspension solution containing RNase A. Then 200 µl of kit lysis buffer was added to the cells; 350 µl of kit neutralization/binding buffer was added to the cell debris for precipitation and separated by centrifugation at 3,000 g for 1 minute. Finally, the plasmid DNA was eluted with a 50 µl elute solution supernatant was passed through a column provided in the kit.

2.13 Gene amplification transformation and replacement

pFA6a-*kanMX6* was the plasmid used as template DNA for amplification (Baehler *et al.*, 1998). Deleted Tsn-kan-F and Tsn-kan-R primers pair was used for *tsn1*. The 50 µl PCR reactions were mixed in a 0.5 ml PCR tube consisting of 1 µl of Expanded High Fidelity polymerase enzyme (Finnzyme), 1 µl of the template DNA (0.02 µg or 20 ng of plasmid DNA), 1 µl of 10 x dNTPs, 1 µl of 20 ng/µl each of forward and reverse primers, 4 µl of Expand Buffer, 3.5 µl of 25 mM MgCl₂ and 36.5 µl of sterile distilled water. The PCR cycles were as follows: denaturing, started at 94°C for 2 minutes, 25 cycles; 94°C for 1 minute; annealing, 55°C for 1 minute; extension at 72°C for 2 minutes; finishing at 72°C for 4 minutes. Ten reaction mixtures were pooled in a 1.5 ml Eppendorf tube, and 5-10 µl of the product was run in a 1% agarose gel to visualise product size. Then 250 µl of phenol

and 250 μ l of chloroform were added and gently vortexed. The tubes were then centrifuged at 3,000 g for 5 minutes (Eppendorf centrifuge 5415D). The clear top layer was aspirated and transferred into a fresh Eppendorf tube. Twice the volume of ice cold ethanol and 0.01 M (final concentration) of NaCl were added, mixed, and the tube was incubated at -20°C overnight or at -80°C for one hour. The tubes were centrifuged at 13,000 r.p.m. for 15 minutes at 4°C in a microcentrifuge. The supernatant was carefully aspirated, leaving the DNA pellet behind. Then 1 ml of 70% ethanol was gently added and drained out. The tubes were left open to air dry. The pellet was resuspended in 20 μ l of TE. Then 1 μ l of the DNA concentrate was run in a 1% agarose gel. The concentration of DNA was determined using a spectrophotometer at a wavelength of 260 nm. Of this DNA, 10 μ g was used for the transformation of *S. pombe* cells, where the concentration of Kan and Nat in YEA is around 100 μ g/L.

Table 2.2 Primers list.

Primer designation	Sequence	Description
Tsn-kan-F	5'-TTA TTT GCA TAC TGA AAA CAT CAT TCG AAT ATC AAC ACT ACT CAA CAG CAT ACA TTA CAG ATT AAG TCG ACG GAT CCC CGG GTT AAT TAA-3'	Forward primer for the kanamycin cassette for <i>tsn1</i> replacement
Tsn-kan-R	5'-ATA TTA AAA AAG CAA TTT TAT CGG CTC AAT TTT AGT CAA GCG TAC AGC TGG CAA ATA AAT TGT TAG CAA TGA ATT CGA GCT CGT TTA AAC-3'	Reverse primer for the kanamycin cassette for <i>tsn1</i> replacement
Tsn-F	5'-GAT CTA AAC AAC CCA AGC G-3'	<i>tsn1</i> ORF Upstream
Tsn-R	5'-GCA TTC ATC ATA GGA CTG CC-3'	<i>tsn1</i> ORF Downstream
Tsn-intC-F	5'-TCA TAT TCC ATT TGA GGC CC-3'	Checking primers for Translin (internal primers)

Screen-tag-F	5'-CAG TTC TCA CAT CAC ATC CG-3'	Placed within kan cassette
Screen-correct-R	5'-CGG ATG TGA TGT GAG AAC TG-3'	Placed within kan cassette
Scrkan 1458-R	5'-CGA CAG CAG TAT AGC GAC CA-3'	Placed within kan cassette
Tsn-1-F	5'-TAT CAA CAC TAC TCA ACA GC-3'	Checking primers for Translin (internal primers)
Tsn-2-R	5'-TTT GAA AAG ATC TGA GGA CG-3'	Checking primers for Translin (internal primers)
Tsn-3-F	5'-AAA CTG ACT GCA GAG GTC G-3'	Checking primers for Translin (internal primers)
Tsn-4-R	5'-CTA ACA AAT CGA CCT CTG C-3'	Checking primers for Translin

		(internal primers)
Tsn-5-R	5'-GCA AAA TTT GAC CAA CTT CG-3'	Checking primers for Translin (internal primers)
Tsn-6-F	5'-CGA AGT TGG TCA AAT TTT GC-3'	Checking primers for Translin (internal primers)
Tsn-7-F	5'-CCC GGT TTT TCC AGA AGA G-3'	Checking primers for Translin (internal primers)
Tsn-8-R	5'-GAA CAC AGA GAT AGT ACT GC-3'	Checking primers for Translin (internal primers)
Tsn-9-R	5'-GAG TTT ACA GAT TGA CGA GC-3'	Checking primers for

		Translin (internal primers)
Tsn-10-F	5'-GCT CGT CAA TCT GTA AAC TC-3'	Checking primers for Translin (internal primers)
Tsn-11-R	5'-CGA AAT GTC TTC TTA AGG AG-3'	Checking primers for Translin (internal primers)
Dcr-pAW1-F	5'- ATAGCTTAGGATTCATTATTTTTTAA GAGACAAATTTCTCGTCAATTGAAT GAAACCTTCCGCCTTTATTTTCTTTT TGACGGATCCCCGGGTTAATTAA-3'	Forward primer of the NAT cassette for <i>dcr1</i> replacement
Dcr-pAW1-R	5'- GCTTTGGAGACCCAAATTGAAAGTT TGAAAAGTTACAAGGGCCGCGGTCA TAAAAAATGAAATACTGTATATTTT AAGTCGAATTCGAGCTCGTTTAAAC- 3'	Reverse primer for the NAT cassette for <i>dcr1</i> replacement
Dcr1 check-	5'- TGATTGAAACTCGAGATGCTTTG	Checking

R	-3'	primers for <i>dcr1</i> : (External primers)
Dcr_int R	5'- GACGATATCATCAGTCACACG-3'	<i>dcr1</i> : Internal Reverse
Dcr_mid F	5'- TCCCTTAGTGTTTGTACAAGC-3'	<i>dcr1</i> : Internal Reverse
Dcr_mid R	5'- TGGCAACTTTACGGGATTTGC-3'	<i>dcr1</i> : Internal Reverse
Dcr1 -F	5'- AGTATTCTGCTCGTGTGATTG-3'	<i>dcr1</i> : External Forward
pAW1-R	5'- ACACGACATGTGCAGAGATGC -3'	<i>dcr1</i> : Internal Reverse
pAW1-F	5'- AGTTTAACTATGCTTCGTCGGC-3'	<i>dcr1</i> : External Forward
Ago-AW1-F	5'- GGTTTGGTATATATAAGCTTCCAACC GCCAAAGCGAATTGTCTTCAGCCAA CTCGTCCTTTATGATTCAGAGTGAGT AGGCGGATCCCCGGGTTAATTAA-3'	Forward primer of the NAT cassette for ago <i>1</i> replacement
Ago-AW1-R	5'- TAAGGAAGTAAAAGTTGTGGGCAAT	Revers primer of the NAT

	CCAGTAGTCAATCGTATATCTATTTTC ATTACTTATTGCATGCAATCCATCAA ACAGAATTCGAGCTCGTTTAAAC-3'	cassette for ago <i>I</i> replacement
AgoNAS-F	5'- ACTTATGTTGCGTTTGC GTGC-3'	ago <i>I</i> : External Forward
AgoNAS-R	5'- GCTATCAACAGTGGATAGAGC-3'	ago <i>I</i> : Internal Revers
pAW1-R	5'- ACACGACATGTGCAGAGATGC-3'	ago <i>I</i> : Internal Revers
AgoNAS-R	5'- GCTATCAACAGTGGATAGAGC-3'	ago <i>I</i> : External Forward
pAW1-F	5'- AGTTTAACTATGCTTCGTCGGC-3'	ago <i>I</i> : Internal Revers
Ago_int F	5'- AGGTACTTGTTAGCTTCATTCG-3'	ago <i>I</i> : Internal Forward
Ago_int R	5'- AGTACCGACATTATTGCGATGC-3'	ago <i>I</i> : Internal Revers
Ago_mid F	5'- TCAAACGCAACATGATCCG-3'	ago <i>I</i> : Internal Forward
Ago_mid R	5'- TCTCAACAAAAGTATCATCGGC-3'	ago <i>I</i> : Internal Revers

2.12 PCR checking of gene deletion.

The programme for PCR strains checking was performed by 1 µl of 10% dilution in water of the template DNA extracted from the candidate strains. The reaction was created using a 2 x ReddyMix (BioLine) reagent PCR program: denaturing, started at 96°C for 1 minute, then 30 cycles of 96°C 1 minute, X°C for 30 seconds, 72°C as necessary, then 72°C for 5 minutes final extension. Alternatively, a Phusion High-Fidelity DNA Polymerase (FINNZYME) was used. The PCR cycles were as follows: denaturing, start at 98°C for 1 minute, then 30 cycles of 98°C 10 seconds, X°C for 30 seconds, 72°C as necessary, then 72°C for 5 minutes final extension. According to the primer set, the annealing temperature (X) varied. The extension time was estimated as 1 minute per kb for 2 x ReddyMix and 15 seconds per kb for Phusion High-Fidelity DNA Polymerase.

2.13 Extraction of whole cell protein and Western blots

For whole-cell protein extraction (WCEs), a single colony of *S. pombe* was grown in 50 ml YEL in a 150 ml flask and incubated overnight in shaker at 30°C until the culture was saturated. Then the culture was harvested by centrifuge at 4000 r.p.m. for 5 minutes. The precipitation was resuspended by using 1.5 ml ice-cold STOP buffer (150 mM NaCl; 10 mM EDTA, pH 8; 1 mM NaN₃). The resuspension was transferred to a 1.5 ml microfuge tube (screw lid) and centrifuged at 5,000 r.p.m.s. for 5 minutes in microcentrifuge. After discarding the supernatant and adding a 50 µl RIPA buffer (10 mM Tris-HCl [pH 7.8], 1% Triton-X 100, 0.1% SDS, 2 mM EDTA [pH 8.15], 150 mM Sodium Ortho-Vanadate, complete protease inhibitor cocktail tablets [Roche]), and 0.3 g acid washed glass beads (Sigma). It was flash spun to make sure beads penetrated the pellet every 30 seconds. The sample was placed on ice for a further 30 seconds. The last two steps were repeated and the

cells vortexed for 3 minutes (the tubes were kept on ice as much as possible). Then a 400 μ l RIPA buffer was added and vortexed for a further 30 seconds. The tube was centrifuged at 20,000 g for 20 minutes at 4°C. The supernatant was collected and the pellet was discarded. The tube was centrifuged at 20,000 g for 10 minutes. This produced the supernatant which could be stored at -80 °C

The concentration of the protein was determined by using the Bradford Reagent (Sigma) as per the manufacturer's instructions. A reducing agent was added to approximately 30 μ g samples of protein, and then run on 10–12% sodium dodecyl sulphate-polyacrylamide gels. This was then electro blotted onto Nitrocellulose or a PVDF transfer membrane (Amersham Biosciences, UK Limited, Little Chalfont, UK). The membranes were incubated in 10% non-fat milk with phosphate-buffered saline (PBS) and 0.1% Tween (blocking buffer) overnight at 4°C. All membranes were washed 3 times for 5 minutes in 1 x PBS and 0.5% TWEEN (Sigma). The membranes were incubated for 1.5 hours at room temperature (approximately 20°C), with primary and secondary antibodies in 10% non-fat milk, 1 x PBS and 0.5% TWEEN for 1.5 hrs. The membrane was washed 3 times in 1 x PBS and 0.5% TWEEN. It was incubated with primary antibody solutions at room temperature. The samples were probed by Donkey anti-rabbit IgG-HRP (1/5000) (Santa Cruz Biotechnology). Donkey anti-guinea pig IgG-HRP (1/5000) (Jackson Immuno Research) was used as a secondary antibody to C-terminus anti-Tsn (1/4000) and C-terminus anti-Trax (1/5000). Blots were probed by using the anti- α -tubulin antibody (1/10000) (Sigma-Aldrich [T5168]) and goat anti-mouse IgG-HRP (1/5000) as a secondary antibody (Santa Cruz Biotechnology). An enhanced chemiluminescence ECL procedure (as explained by Roche) was used for blot visualisation (Amersham Pharmacia Biotech).

2.14 Intergenic recombination assay

For intergenic meiotic recombination analysis, an appropriate dilution of spore suspensions was plated on YEA or NB. Appropriate supplements were added to give a viable spore count. The plates were incubated for 5 days at 33°C. YE + guanine (adjusted to pH 6.5 with 1 M HCl and containing 20 mg/ml of guanine dissolved in 0.35 M NaOH) or NB—fully supplemented but lacking adenine—was used for the adenine prototroph count. Then recombination frequency of prototrophs/10⁶ viable spores was then determined, as the viable counts could then be used for both the spore viability assay and the recombination frequency calculation.

2.15 Microscopy

A growing culture was spun for harvesting and fixed in 1 ml of 100% ethanol. It was rehydrated in 100–200 µl of fixed cells by adding it to 50 µl of water. The cells were fixed by heating them at 70°C until dry. A stock of 1 mg/ml calcofluor was prepared in 50 mM sodium citrate and 100 mM sodium phosphate with NaOH added until the calcofluor dissolved. The working concentration was prepared by applying 50 mM Tris pH 9.5 50 µl to the slides and waiting for 5 minutes. A working solution was prepared from 2 ml 50 mM Tris; the slides were then rinsed and dried. Finally, after applying 3–5 µl of DAPI (1 mg/ml) in Vectashield mounting media (Vector Laboratories) the slides were ready for viewing under the microscope.

2.16 Mini chromosome loss assay

A single colony was picked from the YEA media and inoculated in 5 ml YEL overnight (16 hours) until stationary in an incubator shaker. The temperature was optimised at 33°C. The cells were collected by being spun down at 6,000 r.p.m. in a microcentrifuge; the pellets were washed in 1 ml of distilled sterile H₂O and pelleted onto solid media and

spread by using a glass spreader. Serial dilutions were prepared from these cultures and dilutions of 10^{-1} to 10^{-5} . Then the serial solutions were plated on YE solid media at 30°C (for 2–3 days) until micro colonies were seen (~1 mm in diameter). Each strain was repeated by inoculating seven independent whole colonies into universal bottles. These cultures were grown until saturated by incubation at 33°C with shaking. Then with a glass spreader a dilution of 10^{-4} and 10^{-5} was plated out on YE + guanine (adjusted to pH 6.5 with 1 M HCl and containing 20 mg/ml of guanine dissolved in 0.35 M NaOH). The plates were incubated at 33°C for 3–4 days after which the colonies were counted and tabulated. Seven independent cultures were calculated for mutation rates or recombination rates and the median values were generated from the median Ade⁺ colonies based on the total cell number in the culture (Lea and Coulson, 1949).

2.17 Preparation of electrocompetent *E. coli* cells.

The required *E. coli* cells from frozen stock were streaked onto LB agar and incubated at 37°C overnight (16 hours). A single colony was picked from the plate and inoculated in 5 ml of LB broth and incubated overnight in an incubator shaker at 37°C. One ml of the culture was inoculated in 1-litre flasks with 250 ml LB broth. The cells were grown at 37°C in a shaking incubator for growth to an OD⁶⁰⁰ of 0.5-0.75. The cultures were cooled in ice for 15–30 minutes. From this step on, everything was kept on ice. The cells were harvested by centrifugation at 4°C for 10 minutes. The supernatant was aspirated. Then the pellet was resuspended by adding 40 ml of ice-cold sterile distilled water, and the suspension was transferred to sterile 50 ml falcon tubes. The suspension was then harvested by centrifuge at 1,500 g in chilled water and incubated at 4°C for 15 minutes. The supernatant was drained, the cells were resuspended in 50 ml ice-cold sterile distilled water, and the centrifugation was repeated as described above. All supernatant was removed and the cell pellet was resuspended in a volume of 1 ml ice-cold sterile 10% glycerol. Aliquot cells were stored in 1.5 ml Eppendorf tubes at -80°C.

Chapter 3

Analysis of a potential role for Translin in heterochromatin-mediated gene silencing

3.1 Introduction

Translin is a human protein which was identified as it binds to breakpoint junctions of chromosomal translocations associated with development of some human cancers such as leukemia (Aoki *et al.*, 1995; Laufman *et al.*, 2005). It is conserved in eukaryotes and was identified in mouse as the testis brain RNA binding protein (TB-RBP). It can bind to nucleic acids and has been implicated in a range of functions, including DNA repair, genetic recombination and telomere maintenance. It has a strong ability to bind to single-stranded d(GT)_n and d(TTAGGG)_n regions of the genome (Eliahoo *et al.*, 2010; Laufman *et al.*, 2005; Yu and Hecht, 2008). In addition, Translin favorably binds to consensus sequences such as ATGCAG and GCCC(A/T)-(G/C)(G/C)(A/T), which are associated with the breakpoint junction in several cases of chromosomal translocation (Erdemir *et al.*, 2002; Li *et al.*, 2003). In contrast to human Translin, that binds single stranded G-rich RNA and DNA with about equivalent affinity, the *S. pombe* Translin binds with a greater affinity to RNA than to single-stranded DNA. Therefore, *S. pombe* Translin might play a role in the metabolism of RNA (Eliahoo *et al.*, 2010). Translin has a binding partner protein called Translin-associated factor X (TRAX) which has orthologues in all eukaryotes which have Translin protein (Claußen *et al.*, 2006). TRAX is dependent on the interaction with Translin for stability (Cho *et al.*, 2004) and deletion of Translin results reduction of Trax protein although mRNA levels remains the same, indicating a post transcriptional regulation (Jeandling *et al.*, 2008).

The genome of *S. pombe* has regions that are assembled into heterochromatin, including the centromeres, telomeres and mating-type loci where inserted marker genes are repressed

(Ekwall *et al.*, 1999; Allshire *et al.*, 1995). Fission yeast centromeres are large complex and transcriptionally inactive (Javerzat *et al.*, 1999). They span approximately 35-110 kb and are comprised of central parts of unique non-repetitive DNA (*cnt*) sequences that are surrounded by two inverted inner most repeats (*imr*) which share identical sequences. These are further flanked by the outer repeats (*otr*) (Goto and Nakayama, 2011). There is some evidence to indicate that Translin and TRAX function as regulatory proteins in an RNA interference (RNAi) mechanism (reviewed in Jeandling and McFarlane, 2010). RNAi plays a necessary role in maintenance and establishment of centromeric heterochromatin and gene silencing (Grewal *et al.*, 2007). This is mediated in part by methylation of histone H3 Lysine 9. An alternative RNAi pathway results in the destruction of mRNA (Grewal *et al.*, 2007). Translin and TRAX have been implicated in unwinding of dsRNA and the production of siRNAs that guide the RNA-induced silencing complex (RISC) which cleaves RNA that is homologous to siRNAs inside RISC (Liu *et al.*, 2009). To gain further insight into the relationship between the RNAi and Translin we use the facile nature of the *S. pombe* system to explore whether Translin function is associated with heterochromatin maintenance. In this chapter we aim to make Translin and TRAX strains deleted by replacement of the target gene with *KanMX6* (Kanamycin resistance) cassette, then we used test sensitivity to TBZ, and test whether loss of Translin alleviates heterochromatin silencing in centromere by 5-FOA sensitivity, then determine whether Translin deleted strains are affected by TSA. Finally, we have taken the approach of identifying *tsn1* function for sister chromatids segregation by mini chromosomes loss.

3.2 Results

3.2.1 *S. pombe tsn1* gene deletion

Previous work within the group has demonstrated that heterozygous genetic crosses of Translin deletion strains with *tsn1*⁺ strains results in non-Mendelian marker segregation and unexpected post-meiotic phenotypic variance (R. McFarlane, personal communication). This phenomenon remains uninvestigated in any systematic fashion, but it suggests a meiotic haplo-insufficiency for Translin, the molecular basis of which remains unknown. Thus, the generation of new *tsn1*Δ haploid strains via standard meiotic crosses could not be used. For this reason all *tsn1*Δ strains required in this study were generated by making *de novo* deletions of the *tsn1* gene. The strains of *tsn1*Δ mutants were created by using a PCR based gene targeting methods (Bähler *et al.*, 1998). Gene deletion is achieved by replacement of the target gene with *KanMX6* (Kanamycin resistance) cassette. The cassette is generated by PCR using primers with 70 bp of homology to the sequences immediately flanking upstream and downstream of the target gene open reading frame. The primers also have sequences enabling the amplification of the *KanMX6* gene (Fig.3.1 B). The resulting PCR product is approximately 1.8 kb in size. The purified PCR product was chemically transformed into *S. pombe*. To identify the knockout strains, the transformants were selected on media containing G418 to which *KanMX6* confers resistance and the gene replacement was verified by PCR (Fig. 3.1 C). PCR products of the expected sizes for *tsn1* gene replacement were obtained in three separate reactions for all strains. Nested PCRs were also carried out to confirm that no portion of the *tsn1* gene was still present (refer to Fig. 3.1).

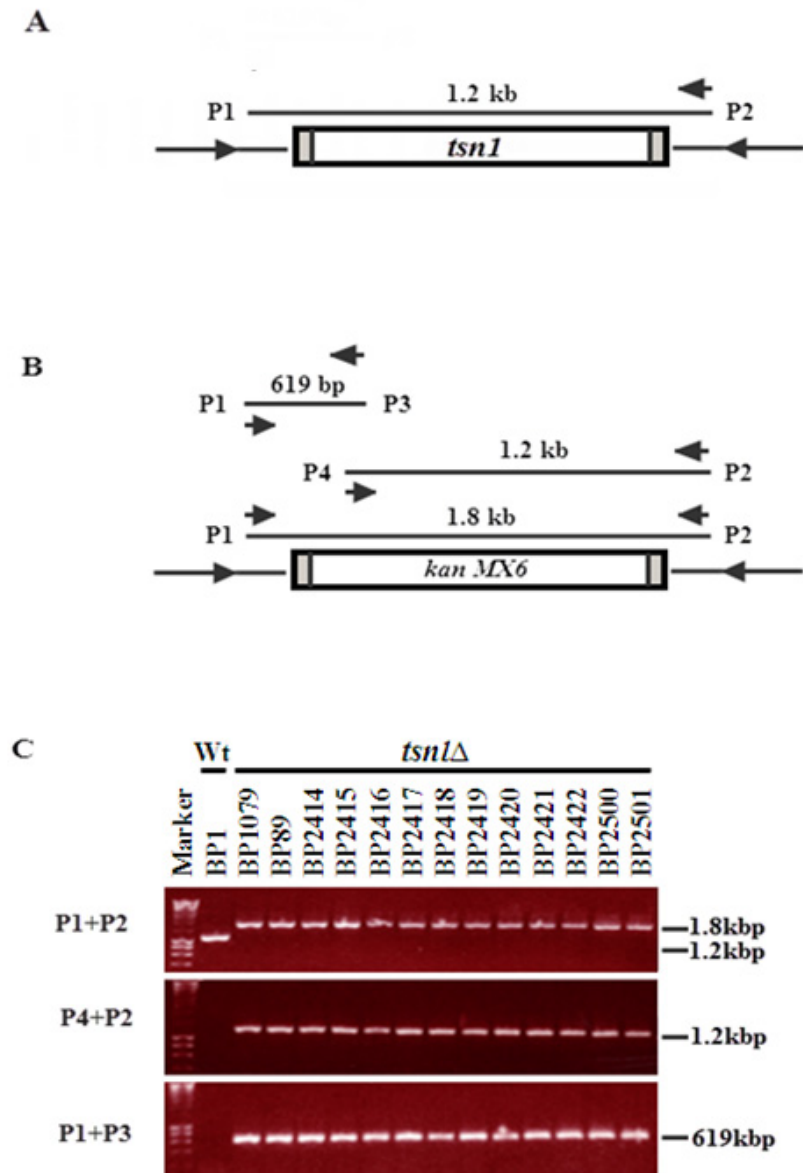


Figure 3.10 PCR results for *tsn1*Δ strains.

A. schematic representation of the sites of the screening primers used and the size of expected PCR product for wild-type. B. The schematic shows the sites of the screening primers used and the size of expected PCR product for knockout strains. C. Imaging of agarose gel showing PCR products by using primers P1 and P2 from wild-type strain and the *tsn1*Δ haploid strains. The expected product size of P1 and P2 in the wild-type strain is ~1.2 kb and ~1.8 kb in the knockout. Primers P2-P4 and P1-P3 produce ~1.2 kbp and 619 bp in *tsn1*Δ respectively, and no product was generated in the *tsn1*⁺ strain, this is further confirmation that *kanMX6* has replaced *tsn1*⁺ in *tsn1*Δ strains.

3.2.2 Western blot analysis of *tsn1Δ* Strains

The selected *tsn1Δ* strains were confirmed by specific PCR of *kanMX6* using specially designed primers. To further confirm the *tsn1Δ* strains western blot analysis was carried out using anti-Tsn1 antibodies (Jaendling *et al.*, 2008). In *tsn1⁺* strains these antibodies detect a band with a mobility of approximately 25 kDa on a 12 % SDS-page gel. No protein was detected in the *tsn1Δ* strains (Fig. 3.2). The stability of Trax is Translin dependent (Jaendling *et al.*, 2008) thus to confirm the *tsn1Δ* strains were functional null mutant the level of Trax was also analysed by western blotting (Fig. 3.3), using anti trax antibodies.

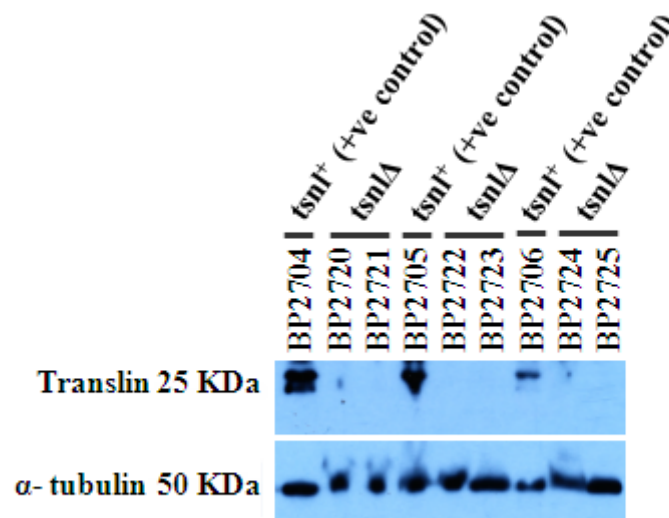


Figure 3.2 Western blot analyses of Tsn1 protein levels in wild-type and *tsn1Δ* strains.

A protein blot of the *tsn1Δ* strains and *tsn1⁺* control strains was probed with anti-Tsn1 antibody. A band migrating with an approximate molecular weight of 25 kDa is shown in the wild-type strains but not in the *tsn1Δ* strains. An anti- α -tubulin blot is shown as a loading control.

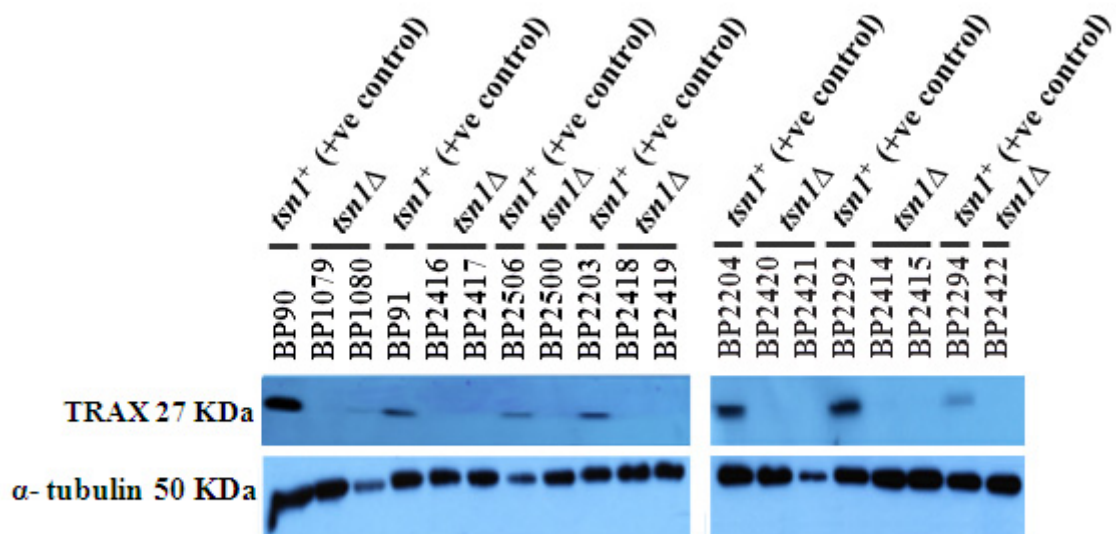


Figure 3.3 Western blot analyses of Trax protein levels in wild-type and *tsn1*Δ strains.

Analysis of Trax protein in the *tsn1*Δ strains. The expected band size of the Trax protein (27 kDa) is seen for the wild-type strains but is not apparent or is reduced in the *tsn1*Δ strains. The Trax protein probed with polyclonal anti-Trax antibodies (Jaendling *et al.*, 2008).

3.2.3 Microtubule inhibitor [thiabendazole (TBZ)] sensitivity tests

Centromeres are heterochromatic regions required to form the link between the chromosome and the microtubules of the mitotic and meiotic spindle apparatus. The microtubule-destabilizing drug thiabendazole (TBZ) has antimitotic activity by inhibiting microtubule functions; cells with defective centromere activity show sensitivity to sub-lethal levels of TBZ. *S. pombe* has been selected as a model system to elucidate the function of Translin, as the RNAi pathway is conserved in *S. pombe* and they have complex, heterochromatic centromeres which require the RNAi pathway for their maintenance. Strains defective in the RNAi pathway are defective in the formation of centromeric heterochromatin capable of mediating transcriptional repression and exhibit sensitivity to sub-lethal levels of TBZ. We set out to establish whether *tsn1Δ* mutants were sensitive to TBZ, which might indicate defective RNAi regulation and defective centromere function. A previous study by the McFarlane group (Jaendling *et al.*, 2008) did not observe any notable TBZ sensitivity in *tsn1Δ* strains, but these experiments were repeated here with new *tsn1Δ* strains to check this earlier observation. To initiate this study new *tsn1Δ* strains were generated (BP1079, BP2720, BP2721, BP2722, BP2723, BP2724, BP2725), which were confirmed by PCR and Western blotting (Fig 3.2 and 3.3). The sensitivity of *tsn1Δ* strain to a range of concentrations of TBZ at different temperatures was tested. *bub1Δ* and *ago1Δ* strains were used as controls (*bub1* encodes the mitotic spindle checkpoint; *ago1* encodes a known component of the RNAi machinery required for centromere function). Extensive analysis of TBZ sensitivities demonstrated that the *tsn1Δ* mutant showed no measurable sensitivity to TBZ, indicating it is not defective in microtubule-mediated chromosome segregation (Fig.3.4).

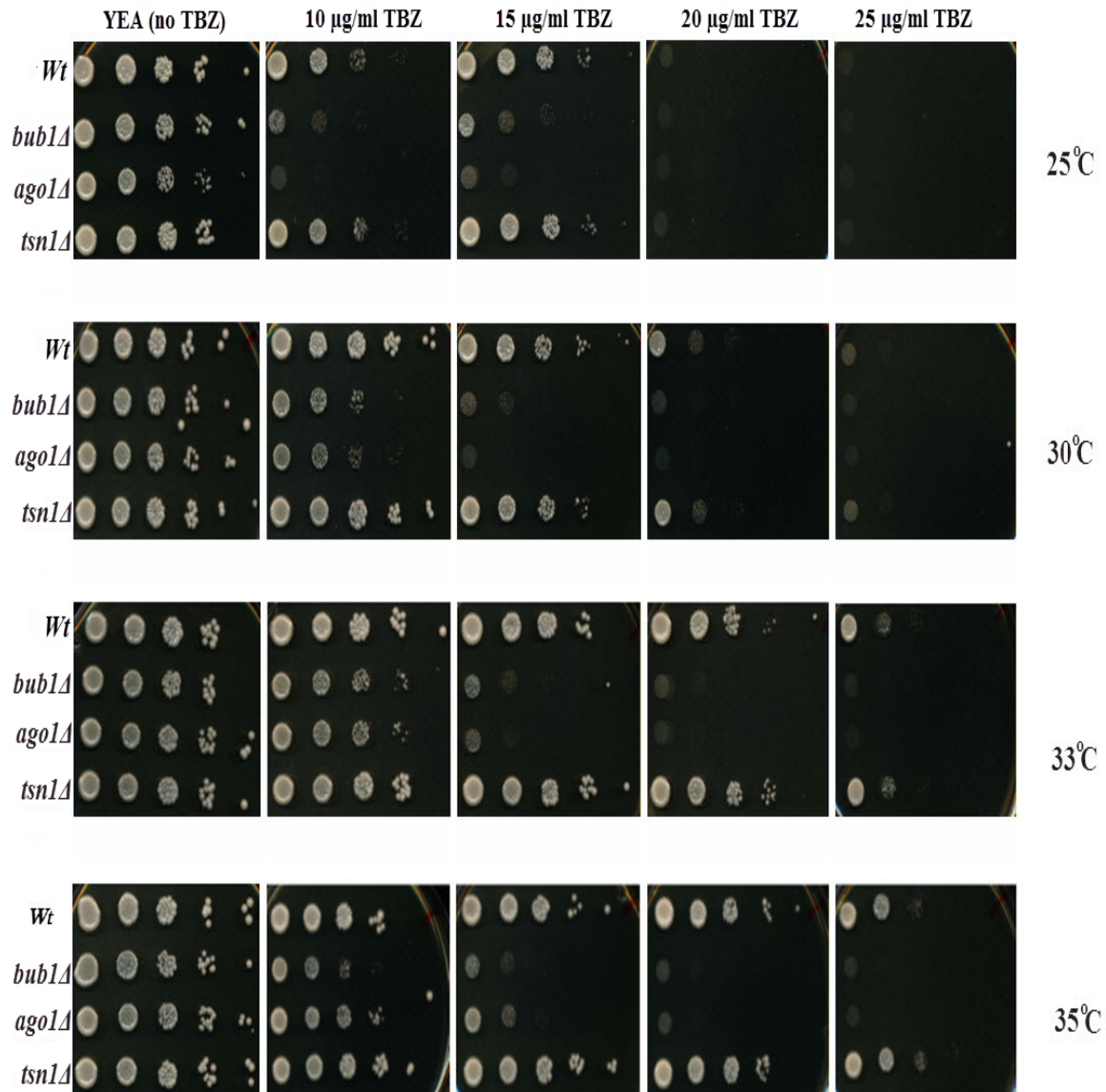


Figure 3.4 TBZ spot test of *tsn1Δ* single mutant.

tsn1Δ(BP1079) was tested for sensitivity to a range of concentrations of the microtubule inhibitor thiabendazole (TBZ). Sensitive to TBZ was tested at a range of temperatures (25°C, 30°C, 33°C, and 35°C) and TBZ concentrations. *bub1Δ* and *ago1Δ* both show increased sensitivity to TBZ relative to wild-type. The *tsn1Δ* mutant (BP1079) shows a similar level of sensitivity to the wild-type at all temperatures and TBZ concentrations.

3.2.4 Analysis of gene silencing in heterochromatic regions.

In the work described in this chapter we used a marker gene, *ura4*⁺, that is inserted in different heterochromatic sites of the *S. pombe* genome (for example, positions of *ura4*⁺ inserted into distinct positions within *cenI* are shown in Fig.3.5). These sites include centromeres, telomeres, rDNA and the mating type loci. These inserts are used to test if Translin controls gene silencing in distinct genome regions. If the *ura4*⁺ is silenced cells can grow on 5-Fluoroorotic Acid (5-FOA). If it becomes de-repressed (active) the cells become sensitive to 5-FOA. 5-FOA sensitivity indicates a defect in heterochromatin-mediated gene silencing.

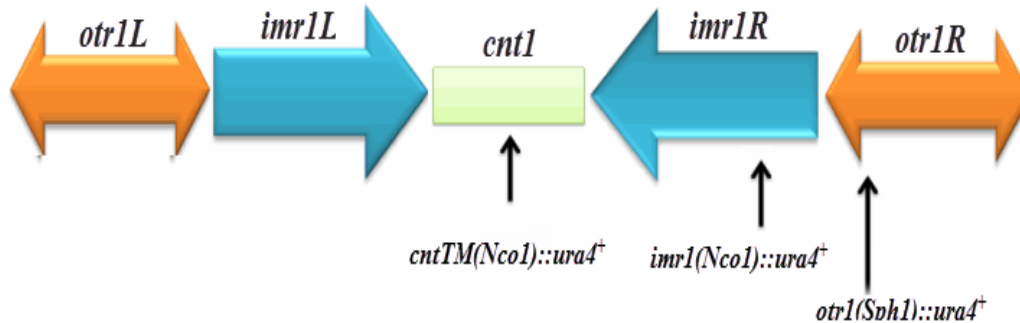


Figure 3.5 Centromeric region of *S. pombe* chromosomes.

The diagram shows the artificial insertion of marker gene *ura4*⁺ into *S. pombe* centromeric heterochromatic region for *cenI*. The diagram shows the sites that the *ura4*⁺ gene has been introduced into in the three distinct heterochromatic sub regions of centromere I (*cenI*) in distinct strains (Adapted from Ekwall *et al.*, 1999).

3.2.4.i Analysis of centromeric gene silencing

Marker genes that are artificially inserted into the *S. pombe* centromeric heterochromatic regions become transcriptionally silent due to RNAi-mediated heterochromatin. The *ura4⁺* gene has been introduced into the three heterochromatic sub regions of centromere I (*cenI*) the *cnt1*, *imr1* and *otr1* in distinct strains (Fig. 3.5). In all cases the *ura4⁺* gene is silenced. Strains with *ura4⁺* deleted at the endogenous locus carrying the centromeric silenced *ura4⁺* gene can grow on 5-FOA but they become sensitive to 5-FOA when heterochromatin-mediated gene silencing becomes deregulated and centromeric *ura4⁺* is expressed. To test whether loss of Translin function alleviates heterochromatin-mediated silencing in centromeres *tsn1Δ* mutants carrying the *ura4⁺* marker located in the three sub regions of the centromeres were generated as described previous (section 3.2.1). Sensitivity to 5-FOA was tested at a range of temperatures (25°C, 30°C, 33°C, 35°C) as centromeric silencing in wild-type cells exhibits a degree of temperature sensitivity (Ekwall *et al.*, 1999). These data demonstrate that there is no difference in the centromeric silencing between the *tsn1Δ* strains as compare with the wild-type strains for all centromeric sub regions (Figure 3.6). An *ago1Δ* mutant was used as a control for strains defective in RNAi-mediated heterochromatin silencing.

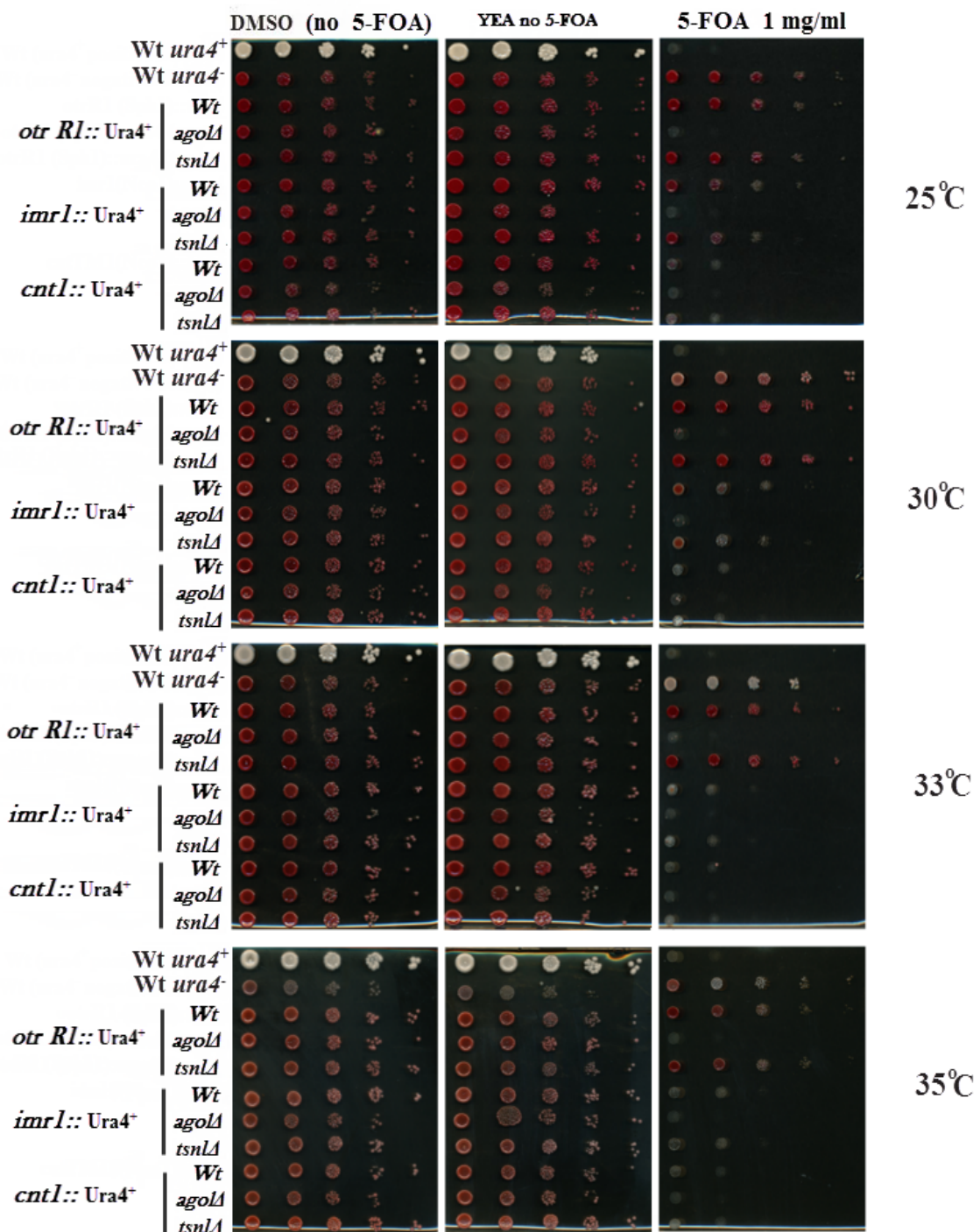


Figure 3.6 Analysis of centromeric gene silencing in *tsn1Δ* mutants.

Wt *tsn1Δ* and *ago1Δ* strains were tested for sensitivity to 5-Fluoroorotic Acid (5-FOA). Plates were incubated at 25°C, 30°C, 35 °C and 35°C for approximately 3 days. *ago1Δ* strains are a positive control as they are defective in gene silencing and exhibit 5-FOA sensitivity. Strains used were (BP1, BP90, BP2506, BP2388, BP2504, BP2388, BP2506, BP2386, BP2501, BP2203, and BP2420).

3.2.4.ii. Analysis of silencing in a *tsn1Δ* mutant at the *rDNA* locus.

Eukaryotic ribosomal RNA genes are located in a specialised region, the *rDNA* locus. The *rDNA* genes are organised as families of long tandem repeats on one or few chromosomes among yeast (Kobayashi *et al.*, 2004). These regions can span from 100-120 tandem repeats in size. The RNA transcript from the *rDNA* constitutes a part of a matured ribosome. Transcription of reporter genes inserted into the *rDNA* locus is silenced due to the heterochromatin nature of this region blocking access to RNA polymerase II (rRNA is transcript by RNA polymerase I) (Dubey, 2009 and Vasiljeva *et al.*, 2008). However, the main role of heterochromatic rDNA may be to avoid the recombination and loss of *rDNA* tandem repeats (Vasiljeva *et al.*, 2008). We employed a strain with *ura4⁺* inserted into a heterochromatic region of the *rDNA* locus to determine whether *tsn1* functioned to regulate *rDNA* heterochromatin-mediated gene silencing. These data demonstrate that there is no difference on media supplemented with 5-FOA effect on *tsn1Δ* strains as compare with the wild-type strains.

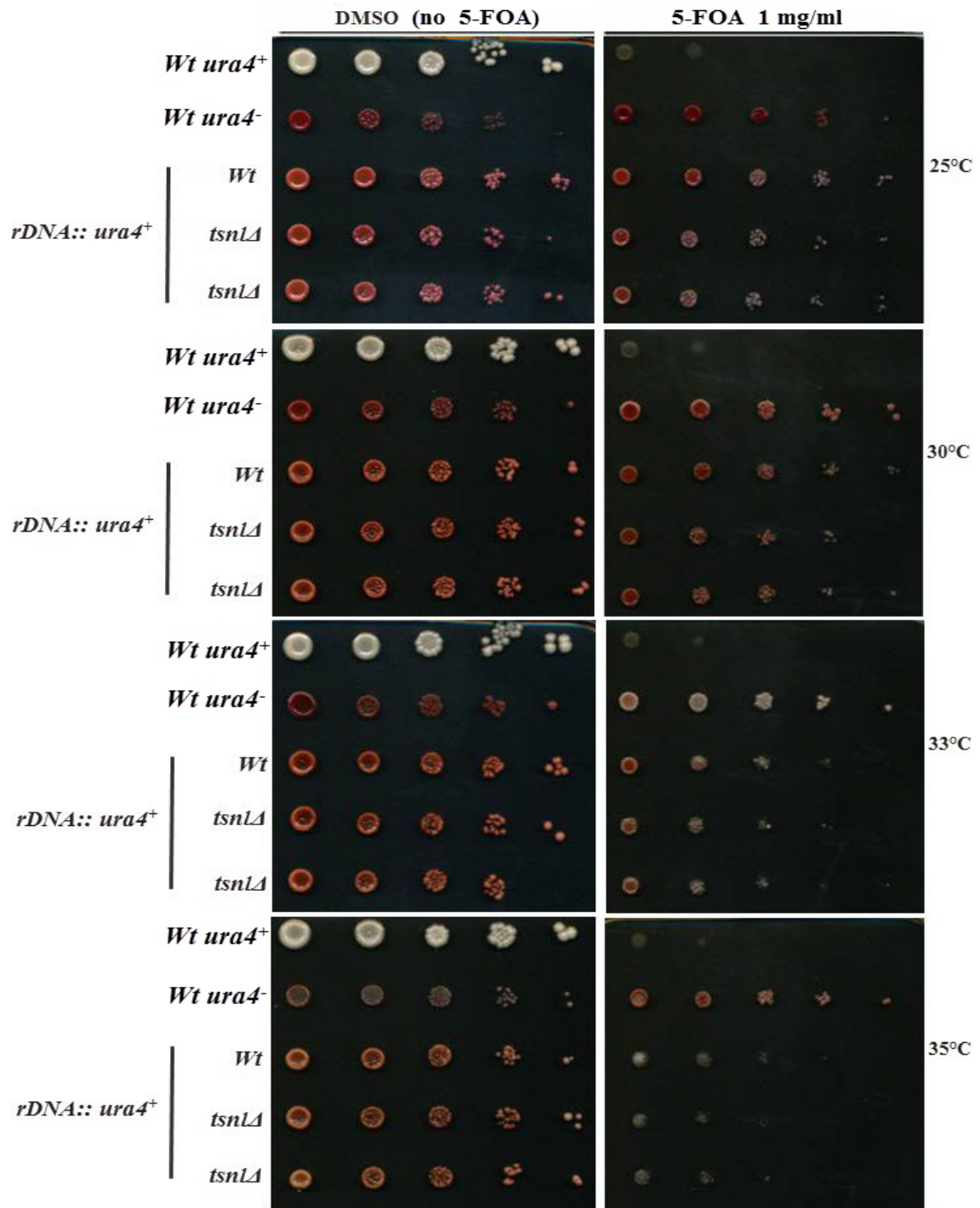


Figure 3.7 Analysis of *rDNA* gene silencing in single *tsn1Δ* mutants.

The results illustrate that the wild-type strains and two independently generated *tsn1Δ* strains have the same sensitivity level to 5-FOA. The plates were incubated at 25°C, 30°C, 33°C and 35°C. Strains used were (BP1, BP90, BP2704, BP2720, and BP2721).

3.2.4.iii. Analysis of silencing in a *tsn1Δ* mutant at the mat locus.

Mating is a necessary step for a sexually reproducing organism. The mating-type locus in haploid cells of *S. pombe* is a characterised heterochromatic region. The *S. pombe* mating-type loci are comprised of three cassettes: *mat1*, *mat2P* (h^+) and *mat3M* (h^-) (Fig. 3.8). Both *mat2P* (h^+) and *mat3M* (h^-) are transcriptionally inactive (Hansen *et al.*, 2011; Vengrova and Dalgaard, 2004); whilst the *mat1* locus which contains the genetic information of P or M is transcriptionally active, and determines the cell mating-type (Hansen *et al.*, 2011). Upon starvation of nitrogen, switching occurs by transpositions of DNA by a recombination between the silent loci *mat2* or *mat3*, into *mat1* (Thon and Klar, 1992). Therefore, h^+ mating-type locus cells produce P pheromones and make M pheromone receptors for the opposite pheromone on the cell surface and *versa vice* (Klar, 2007). In the presence of an appropriate mating partner the opposite kinds of haploid cells will conjugate to form a zygote following meiosis. The mating-type locus elements are targeted by RNAi to recruit chromatin modifiers and establish silent heterochromatin (Bayne *et al.*, 2010). Heterochromatin in these sites is specified by H3K9me, and the presence of chromodomain proteins and hypoacetylation of histones (Hansen *et al.*, 2011). *cenH* which is between *mat2-P* and *mat3-M*, (Fig. 3.9) is homologous to centromeric repeats and produces siRNAs and non-coding RNAs. These non-coding RNAs have the ability to guide the RNAi factors to somehow facilitate the H3K9me establishment. However, in mating-type loci RNAi is not necessary for H3K9me, since mutant of a vital RNAi component such as *dcr1*, *ago1* are not distinguishable from wild-type (Hansen *et al.*, 2011). This is because in fission yeast there are two pathways responsible for gene repression and heterochromatin formation within the mating-type at (*mat2/3*). One is a siRNA-dependent pathway (see above), and the other is siRNA-independent pathway (Kim

et al., 2004; Yamada *et al.*, 2005). The activating transcription factors Atf1 and Pcr1 which can bind Clr4, Swi6 and the histone deacetylase Clr6 are important for deacetylation of both H3 and H4, where this step is very important for subsequent H3 lysine 9 methylation histone by Clr4 (Kim *et al.*, 2004). We tested whether loss of Translin has an influence on mating-type silencing. To do this we used a strain background with the *ura4*⁺ marker inserted into heterochromatic regions which render it silent.

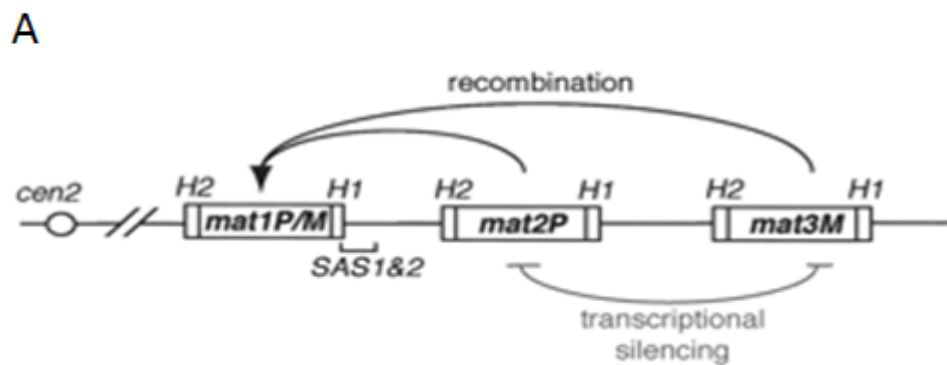


Fig. 3.8 Diagram of mating type locus of *S. pombe*.

The diagram shows the mating type locus of *S. pombe* on chromosome 2. *mat2P* and *mat3M* cassettes are shown. The black arrows show the recombination occurrence, that exchange the cassette at *mat1* with the opposite mating-type loci information (adapted from Verdel and Moazed, 2005)

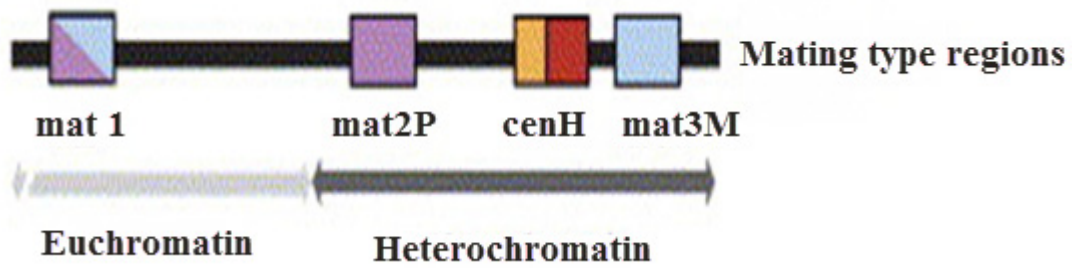


Fig.3.9 Mating type region domains

The regions of mating-type loci contain a domain called *cenH*, its size is around 3 kb and has identity of more than 96% with the *dh/dg* repeats of the centromeric *otr* repeats. Adapted from (Verdel and Moazed, 2005).

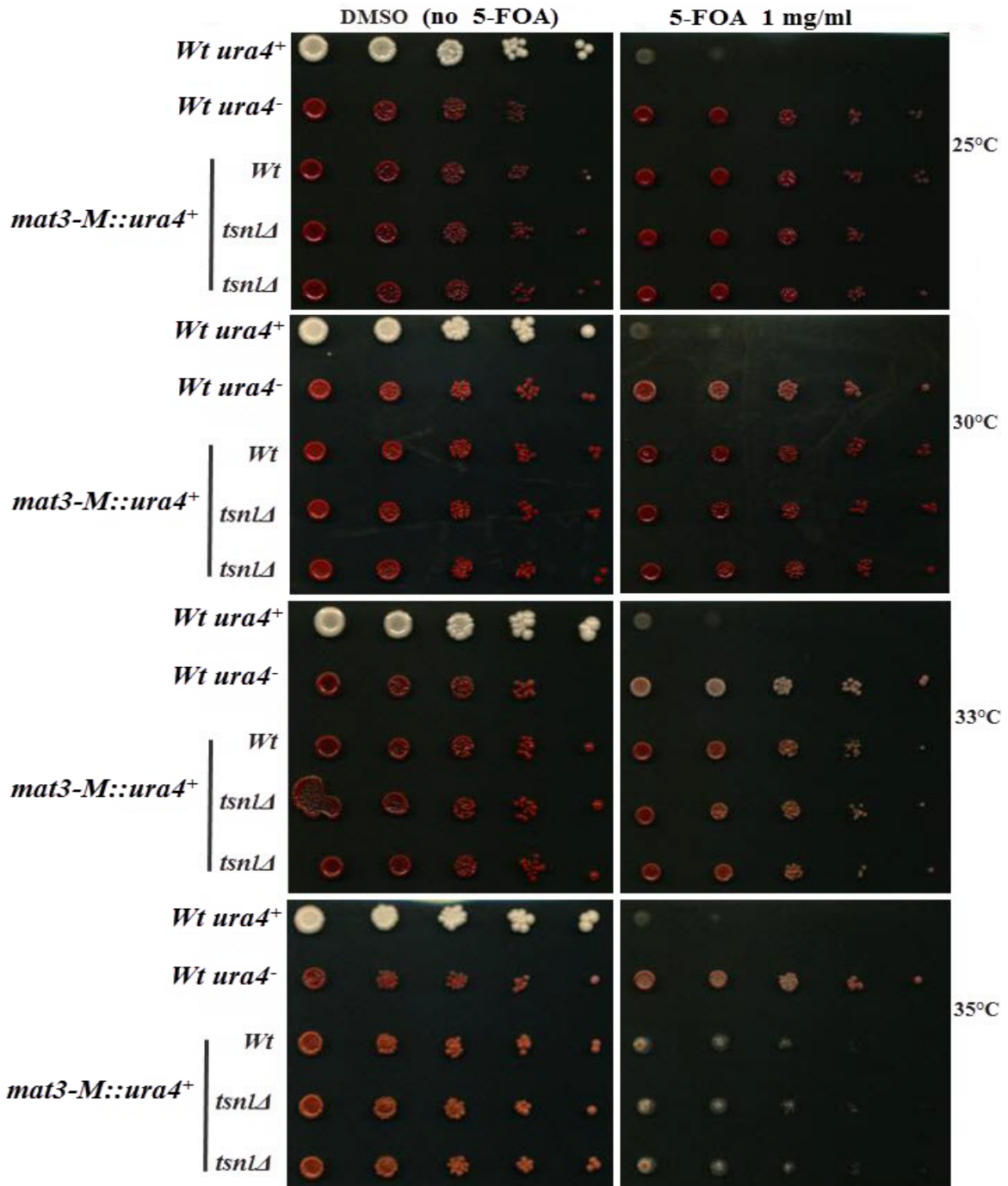


Figure 3.10 Analysis of *mat3-M* gene silencing in single *tsn1Δ* mutants

Dropped culture of *Wt* and *tsn1Δ* strains which plated on supplemented YEA at different temperature 25°C, 30°C, 33°C and 35°C. The data show no difference in sensitive between the wild-type and *tsn1Δ* strains. (BP1, BP90, BP2706, BP2724, and BP2725).

3.2.4.iv. Analysis of gene silencing in subtelomeric heterochromatin in a *tsn1Δ* mutant.

The ends of linear chromosomes in eukaryotic cells are stabilised by specialised DNA proteins (nucleoproteins) complex termed telomeres (Bah and Azzalin, 2012). The telomeres are fundamental for chromosome stability and for preventing loss of genetic material at chromosome ends during replication and they also avoid chromosomal rearrangement or fusing of chromosome to each other (Xin, 2008; Mandell, 2005). During the replication of DNA the telomeric DNA length become shorter but it is maintained and renewed by the enzyme telomerase (Ukimori *et al.*, 2012). In mammals as well as in *S. pombe* the ends of telomeric DNA contain tandem substrate repeat sequences of DNA, where telomeric repeat in vertebrates are 5'-TTAGGG-3' elements, and in *S. pombe* with some variations in the length of G tracts, 5'-TTAC(A)GG(G1-4)-3' sequence (Bah and Azzalin, 2012; Chikashige *et al.*, 1997). The extensions for vertebrates and *S. pombe* are 2-50 kb and 300 bp respectively (Bah and Azzalin, 2012). The subtelomeric regions are also heterochromatic and are enriched for repetitive sequences; genes placed into these regions are also transcriptionally silenced, a phenomenon called telomere position effect (Blasco, 2007; Allshire *et al.*, 1995; Ekwall *et al.*, 1995; Bah and Azzalin, 2012). Here we employed a *ura4⁺* marker gene inserted into the sub-telomeric heterochromatic region to determine whether Translin functions to maintain the telomere positions effect. We did not observe any obvious decreased growth on media supplemented with 5-FOA effect on Translin deleted strains (Fig. 3.11).

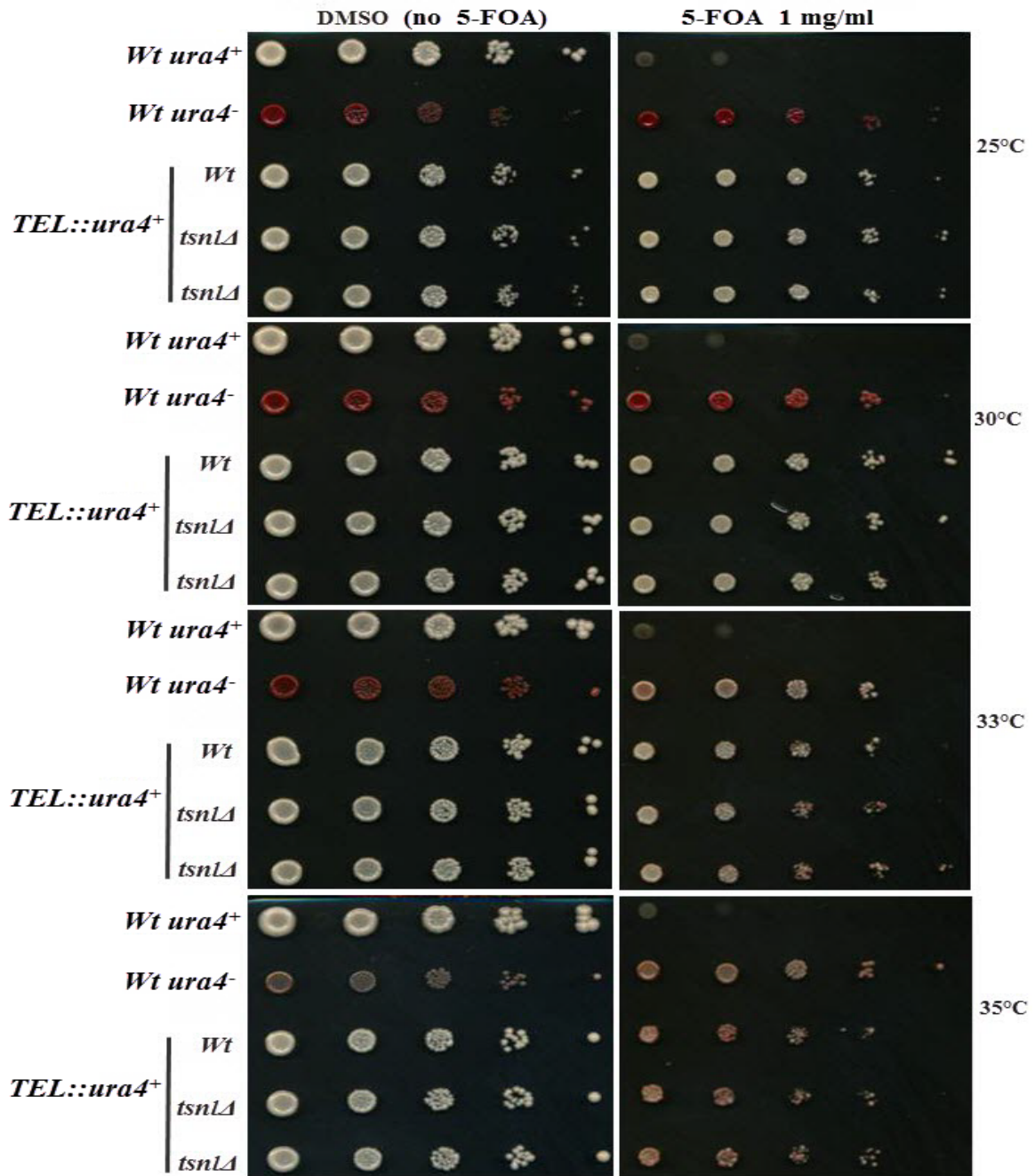


Figure 3.11 Analysis of *TEL* gene silencing in single *tsn1Δ* mutants.

At all temperatures tested the wild type and *tsn1Δ* show no difference in the sensitivity to 5-FOA. (BP1, BP90, BP2705, BP 2722, and BP2723).

3.2.5 TSA sensitivity tests

TSA is HDAC (histone deacetylases) inhibitor which consider as class of anticancer agents (Alao *et al.*, 2009; Kimata *et al.*, 2008). HDACs have an essential function in heterochromatin formation and other epigenetic regulatory pathway (Kimata *et al.*, 2008). To determine whether Translin deleted strains are affected by TSA, spot tests were employed. As shown in figure 3.12 *tsn1Δ* exhibited no increased sensitivity to TSA relative to the wild-type control, indicating it is not sensitive to mild HDAC inhibition. *mis6-302* is a positive control and *rec10:: KanMX6* and *hop1:: KanMX6* strains were used to ensure there was no influence on TSA sensitivity due to the *KanMX6* cassette (*rec10/ hop1* are meiosis-specific genes with no mitotic function). Slight sensitivity was seen in the isogenic wild-type strain (BP90) which is thought to be due to marker gene defects, which are suppressed by the introduction of the *KanMX6* cassette as both *rec10:: KanMX6* and *hop1:: KanMX6* show the same suppressed as the *tsn1:: KanMX6* strain. A *Trax:: KanMX6* shows no increased sensitivity, but the function of the *Trax* gene was not a major goal of this study.

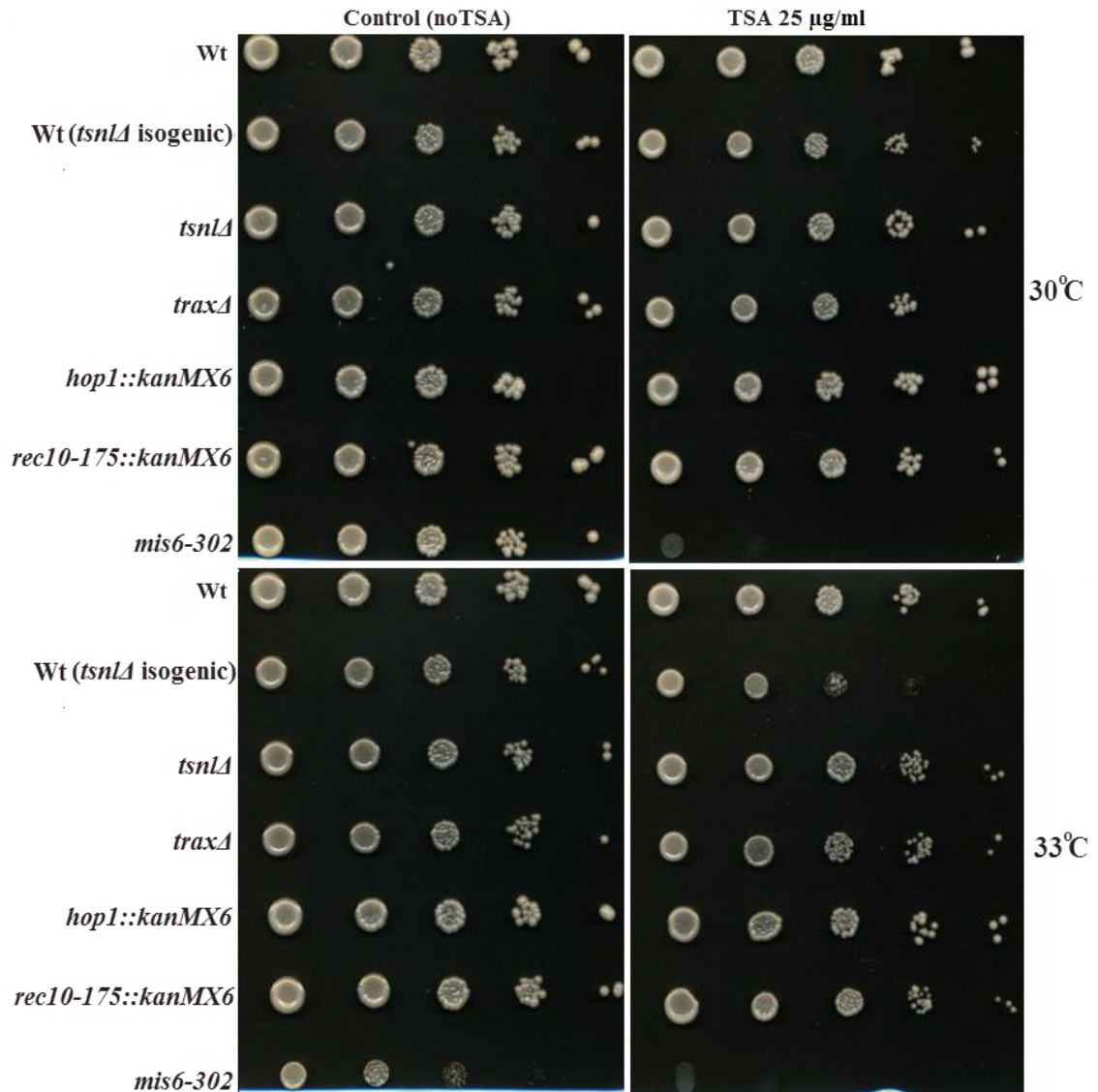


Figure 3.12 Spot tests of effect of TSA on the growth of wild-type, *mis6-302* (positive control), *tsn1Δ*, *trax1Δ*.

These strains were grown at 30°C and 33°C, on YEA with supplements, these dishes lacking or containing TSA (25 µg/ml). The *tsn1Δ*, *trax1Δ* strains show no sensitive to the TSA. (BP1, BP90, P1079,1090,BP153 ,BP1731 ,BP846)

3.2.6 Minichromosome Loss Assay

The centromere DNA which consists of central and outer heterochromatin play an essential function in accurate segregation of chromatids, and confirm faithful equivalent distribution of cell chromosomes throughout cell division in eukaryotic cells (Yanagida, 2005; Saitoh *et al.*, 1997; Baum *et al.*, 1994). Therefore, accurate centromere interaction with the mitotic spindle is vital for precise chromosome separation, and centromere instability can lead to chromosome loss and gain events (Saitoh *et al.*, 1997; Ekwall, *et al.*, 1999). The central domain and a segment of heterochromatic outer repetitive area are essential for an active centromere and accurate separation (Yanagida, 2005; Volpe *et al.*, 2003) Given that, in *S. pombe*, factors that have a role in centromeric heterochromatin are essential for accurate separation of chromosome, mutation of RNAi regulatory genes alleviate the repression of centromeric marker genes and as well cause missegregation of chromosomes (for more detail refer to Introduction) (Sugiyama *et al.*, 2005; Reddy *et al.*, 2011; Ekwall, *et al.*, 1999). In this chapter, we use a mini chromosome loss assay system in *S. pombe* for centromere function investigation. We have taken the approach of identifying *tsn1* function for sister chromatids segregation by the colony color assay method, by using BP2294 as wild-type, BP 2472 (*ago1:: KanMX6*) as positive control and BP 2421 *tsn1Δ*. Our data shows that the *tsn1Δ* mutation does not have an affect on chromosome segregation in an otherwise wild-type background (Fig. 3.13).

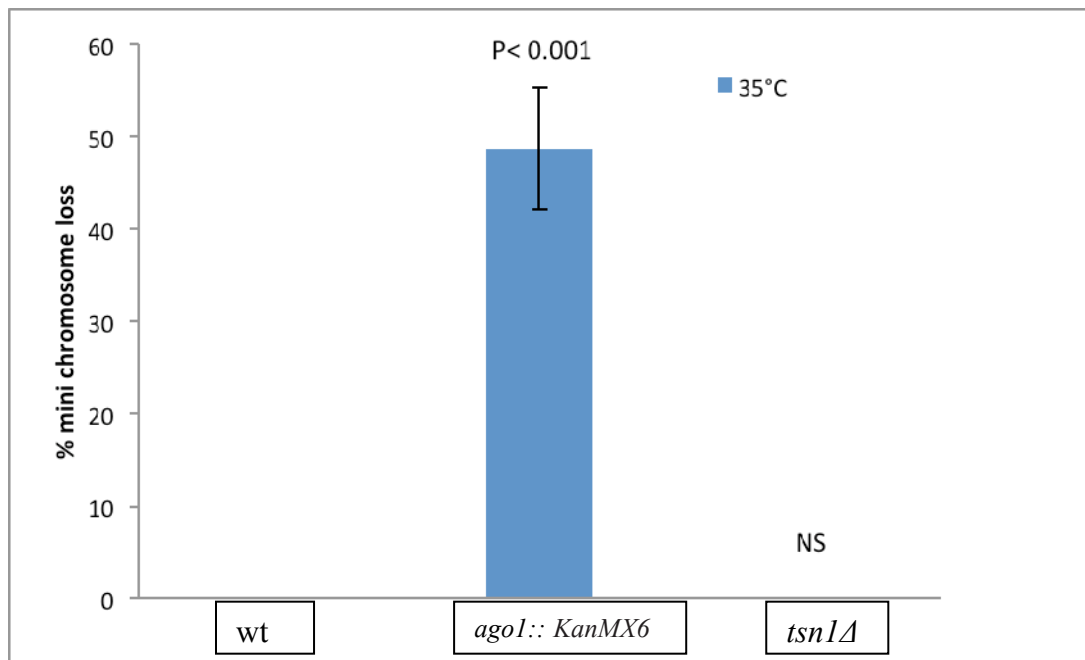
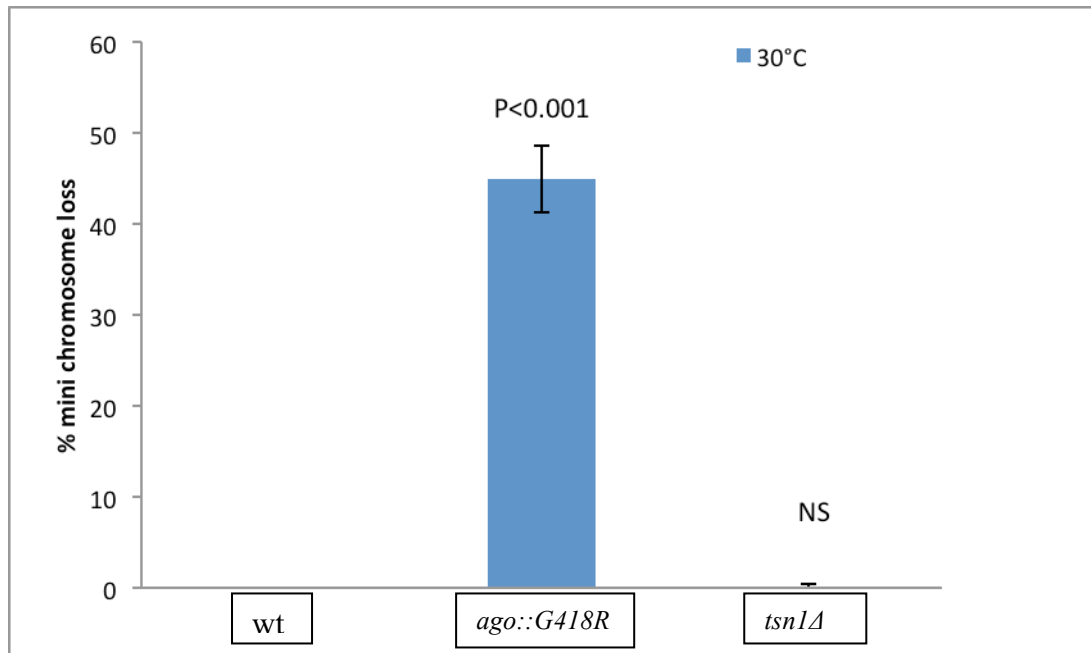


Figure 3.13 Mini chromosome loss in *tsn1Δ* single mutant.

Histograms are mean values of *tsn1Δ* and a positive control *ago1::KanMX6* median values derived from 3 repeats of 7 independent samples. Culture was tested at 30°C (top) and 35°C (bottom). Average colonies counted 30°C: wt (2054), *tsn1Δ* (1776) and *ago1Δ* (1681) colonies. 35°C: were wt (2086), *tsn1Δ* (1709), *ago1Δ* (2054) respectively. Error bars are standard deviations. *P* values were derived by Student's *t*-test and represent pair-wise comparisons between wild-type and mutant (*tsn1Δ* or *ago1Δ*). NS= No significant.

3.3 Discussion

3.3.1 *tsn1* gene deletion

Translin is an evolutionarily conserved 25 kDa and it may be involved in various biological processes (Jeandling and McFarlane, 2010). Translin has a binding partner protein known as Translin-associated factor X (Trax) 27 kDa which forms a specific complex with Translin, and Trax is depend on Translin for its stability, so in the absence of Translin, Trax protein is reduced (Jeandling *et al.*, 2008). We have successfully isolated new *tsn1*Δ strains by using a PCR based deletion strategy (Bähler *et al.*, 1998). These strains were confirmed by using PCR and western blotting methods. Which show that the *tsn1*Δ genes in these strains were deleted entirely. This strategy of mutant development was used extensively during the remainder of this study.

3.3.2 *tsn1*Δ single mutant strains are not effected by the microtubule inhibitor thiabendazole (TBZ)

In confocal microscopic studies of the cell cycle in Human Embryonic Kidney (HEK) cells it was found that in prophase stage Translin was located at the centrosomes and in metaphase it was located on the mitotic spindle. After that it moves to the spindle midbodies throughout cytokinesis. This observation shows Translin may be implicated in processing of chromosome segregation and cytokinesis (Ishida *et al.*, 2002), and that Translin associates with microtubules (Ishida *et al.*, 2002). Therefore, Translin may have a role for microtubules stabilization. In *S. pombe* Translin single mutant cells, we found no increase sensitivity to the microtubule inhibitors TBZ. This suggests that if there is a role in microtubule dynamics or centromere function it is not essential or is redundant.

3.3.3 Centromeric gene silencing in *tsn1Δ* mutants.

Centromeres are essential regions for chromosomes segregation and they interact with the spindle microtubules (MTs) (Pidoux and Allshire, 2005). The heterochromatin regions of centromeres in *S. pombe* are transcriptionally silent insertion of marker genes results in transcriptional repression (Pidoux and Allshire, 2005). The centromere is the essential component for accurate chromosome inheritance (Nechemia-Arbely *et al.*, 2012). Deviation of centromeres by deregulation of centromeres structure is a character of several human cancers. Given that the formation of heterochromatin silencing in centromere outer repeat regions depends on the RNA interference machinery, we hypothesised Translin may have a role for RNAi- mediated gene silencing at centromeres given the fact it has been implicated in RNAi control (Liu *et al.*, 2009). However, in Translin single mutant strains, we found no loss of gene silencing function. This suggests there is no role for Translin in centromeric gene silencing, or it has a redundant role.

3.3.4 No role of *tsn1Δ* single mutant in mating loci, rDNA and telomeres gene silent regions

Mating loci (HM), telomeres, and rDNA repeats are transcriptionally silent regions (Michael *et al.*, 2011). So the gene marker inserted inside these regions is important for study silencing and heterochromatin. The growth of *tsn1Δ* strains on media with 5-FOA was similar to wild-type. This finding may imply that Translin does not have any crucial role in maintaining or generating silencing in these regions. Thus, the data obtained in this chapter provide no functional link between Translin and a role in RNAi-mediated gene silencing/heterochromatin formation. However, in subsequent chapters a possible redundancy of function is investigated further.

3.3.5 *tsn1Δ* mutant does not cause increase TSA sensitivity tests

A histone deacetylase (HDAC) inhibitor, trichostatin A (TSA) is kind of anticancer agent (Kimata *et al.*, 2008). In this study we used *S. pombe* as a model because it contains three HDACs similar to mammalian organisms (Kimata *et al.*, 2008). HDACs have a role in heterochromatin formation and maintenance. TSA can cause disturbance of centromeric heterochromatin in addition to loss of chromosome. (Kimata *et al.*, 2008). Our results show no sensitive in *tsn1Δ* strains for TSA. This further supports the notion that Translin does not provide a primary pathway for the establishment/maintenance of heterochromatin.

3.3.6 No effect on chromosomes segregation stability

Chromosomes contain distinct functional domains, which confirm faithful equivalent distribution of chromosomes throughout cell division (Saitoh *et al.*, 1997; Baum *et al.*, 1994). The centromere DNA has a essential function in correct sister chromatid segregation (Saitoh *et al.*, 1997) where, the centromere serves as an attachment point for spindle microtubules, and form kinetochores by interact with several proteins (Roay and Sanyal, 2011). In *S. pombe* the centromeres are linked with arrays of repetitive DNA nucleotides, where the outer repeat is heterochromatic and are needed for an active centromere (Volpe *et al.*, 2003). The transcripts of these repeats uses the RNAi machinery for creation and mediation of heterochromatin assembly (Volpe *et al.*, 2003). Therefore mutation of RNAi proteins results to impair silencing and centromere function (Sugiyama *et al.*, 2005; Reddy *et al.*, 2011). The data in this chapter does not show a direct link between *tsn1Δ* and missegregation of chromosome and heterochromatin function. In the following chapter a redundant role in chromosome stability with RNAi pathway is investigated.

3.4 Conclusions

- 1- Translin deleted *S. pombe* strains were successfully generated by using the PCR based gene deletion technique and confirm by western blotting (Bähler *et al.*, 1998).
- 2- Single deletion of Translin demonstrates no temperature sensitivity to TBZ.
- 3- Regions of heterochromatin exhibit no loss of gene silencing function in *tsn1Δ* strains.
- 4- Loss of Translin does not result in measurable chromosome instability.

Chapter Four

Analysis of Translin and RNAi-defective double mutants

4.1 Introduction

During mitosis sister centromeres capture the spindle microtubule via a multiprotein complex called the kinetochores (Cleveland *et al.*, 2003). At anaphase the attached sister-chromatids separate towards the nuclear poles, resulting in identical daughter cells (Roay and Sanyal, 2011; Mythreye and Bloom, 2003). Mitotic and meiotic chromosome segregation is a high fidelity process, where faithful and equal distribution of chromosomes depends on a many process, such as the repair of DNA, chromosome condensation, correct arrangement of the spindle and sister chromatid segregation (Roay and Sanyal 2011; Javerzat *et al.*, 1996). Therefore, abnormal separation of mitotic chromosomes results in genomic instability and is associated with cell proliferative diseases, such as cancer (Ishida *et al.*, 2002). *S. pombe* centromeres require repetitive sequences for active centromeres, where the outer repeats (*otr*), composed of *dg* and *dh* elements are heterochromatic in nature (Volpe *et al.*, 2003; Kagansky *et al.*, 2009). At these repeats the RNA interference (RNAi) machinery plays an a crucial role in heterochromatin assembly, assisting accurate separation of chromosomes (Volpe *et al.*, 2003; Sugiyama *et al.*, 2005; Reddy *et al.*, 2011). Therefore, deletions of RNAi component genes, for example, Argonaute (*ago1*), Dicer (*dcr1*) or RNA-dependent RNA polymerase (*rdp1*) impair heterochromatin-mediated silencing and centromere function (Reddy *et al.*, 2011; Sugiyama *et al.*, 2005). Consequently, these mutants leads to increased loss of chromosomes, lagging chromosomes on late anaphase spindles and hypersensitivity to microtubule-destabilizing agents such as thiabendazole (TBZ) (Pidoux and Allshire, 2005; Reddy *et al.*, 2011).

A minichromosome loss assay system is useful to study *S. pombe* centromeric function, where a minichromosome carries a whole *S. pombe* centromeric DNA region which is a mitotically stable (Reddy *et al.*, 2011). In the case of RNAi mutants, like *dcr1Δ*, *S. pombe* cells lose pericentric heterochromatin function, resulting in chromosome missegregation and minichromosome instability (Reddy *et al.*, 2011; Volpe *et al.*, 2003; Bayne *et al.* 2010 ; Javerzat *et al.*, 1996). In *Drosophila*, the Trax/Translin heteromers, form a complex called C3PO, which may be implicated in siRNA-mediated silencing by activation of RNA-induced silencing complex (RISC), where C3PO has the ability to facilitate endonucleolytic cleavage of the siRNA passenger strand (for more details see the Introduction) (Jaendling and McFarlane, 2010; Liu *et al.*, 2011). Moreover, there is a relationship between the rate of cell division and Translin levels in mammalian cells (Ishida *et al.*, 2002). Confocal microscopic studies have shown that Translin is placed at the centrosomes at prophase; in addition, Translin was detected on the mitotic spindle at metaphase, after that in late telophase Translin migrated to midbodies (Fukuda *et al.*, 2008; Ishida *et al.*, 2002). These findings propose that Translin may have a role in regulating cell proliferation. Other evidence has proposed that Translin may be implicated in the DNA damage response, as the levels of Translin are elevated in nucleus when mammalian cells are treated with DNA damage, for example, ionizing irradiation and oxidative stress (Fukuda *et al.*, 2008; Ishida *et al.*, 2002; Yang *et al.*, 2004; Kasai *et al.*, 1997). However, the role of Translin in the nucleus in response to DNA damaging is unclear. In Chapter 3 we find no loss of heterochromatin silencing, no evidence for increase genome instability or cell division defects in *tsn1Δ* single mutants. However, given Translin role in the C3PO complex of *Drosophila* we speculated that Translin might have functions which are redundant with components of the RNAi machinery. In this chapter this hypothesis is tested by construction and analysing a number of double mutants of *tsn1Δ* and *dcr1Δ/ago1Δ* to determine whether these mutants exhibit defects more severe than *dcr1Δ* or *ago1Δ* mutants alone.

4.2 Results

4.2.1 Strains construction

Given that *tsn1Δ* mutants possibly have a meiotic haploinsufficiency defect which results in progenitor strains with phenotypic abnormalities (McFarlane, personal communication), we made all required double mutants using the direct deletion method (Bähler *et al.*, 1998). *dcr1Δ* and *ago1Δ* null mutants were constructed using the same PCR based gene targeting method. Primers were designed that had 100 bp of homology to the sequence immediately upstream and downstream of the *dcr1* and *ago1* open reading frames (ORF) and 20 bp of homology to the plasmid pAW1 (Watson *et al.*, 2008) which carries the *ura4⁺* gene as the selectable marker for deletion strain construction. The PCR product (Figure 4.1) for the wild-type *dcr1⁺* gene should give a band of roughly 4409 kb and a band of 2155 kb for the knockout strains. For the wild-type *ago1⁺* gene the PCR product sizes should be 3322 kb and 2317 kb for the knockout strains (Figure 4.2). The whole homologous cassette (Bähler *et al.*, 1998) was chemically transformed to a *S. pombe* haploid strain (BP90), which carry the background markers *h⁻ ade6-M26 ura4-D18 leu1-32* for single *dcr1Δ* or *ago1Δ* and also into *tsn1Δ* null mutant strain BP1079 for double mutants strains (*tsn1Δ dcr1Δ*) and (*tsn1Δ ago1Δ*).

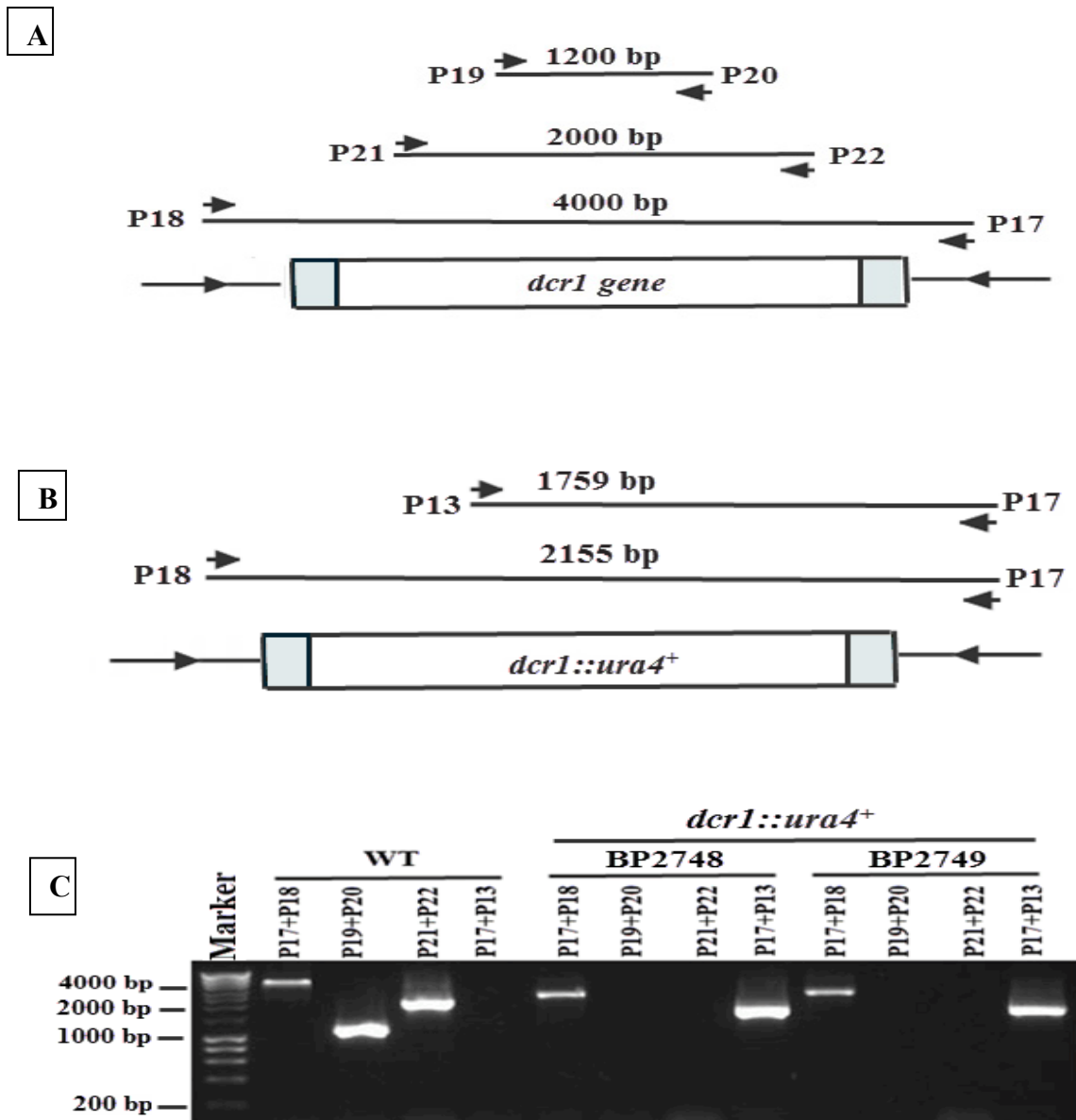


Figure 4.1 PCR results for *dcr1Δ* strain construction.

A. Schematic illustration of the sites of the screening primers used and the size of expected PCR product for wild-type. B. The schematic demonstrates the sites of the screening primers used and the size of expected PCR product for knockout strains. C. DNA from the candidate *dcr1Δ* strains subject to a variety of PCR primers where imaging of agarose gel viewing PCR products by using primers P17 and P18 from wild-type strain and the *dcr1Δ* haploid strains. The expected product size of P17 and P18 in the wild-type strain is ~4000 bp and ~2155 bp in the knockout. Primers P21-P22 and P19-P20 produce ~2000 bp and ~1200 bp in wild-type respectively, and no product was generated in the *dcr1Δ* strain, this is further confirmation that *ura4⁺* has replaced the *dcr1* in *tsn1Δ* strains.

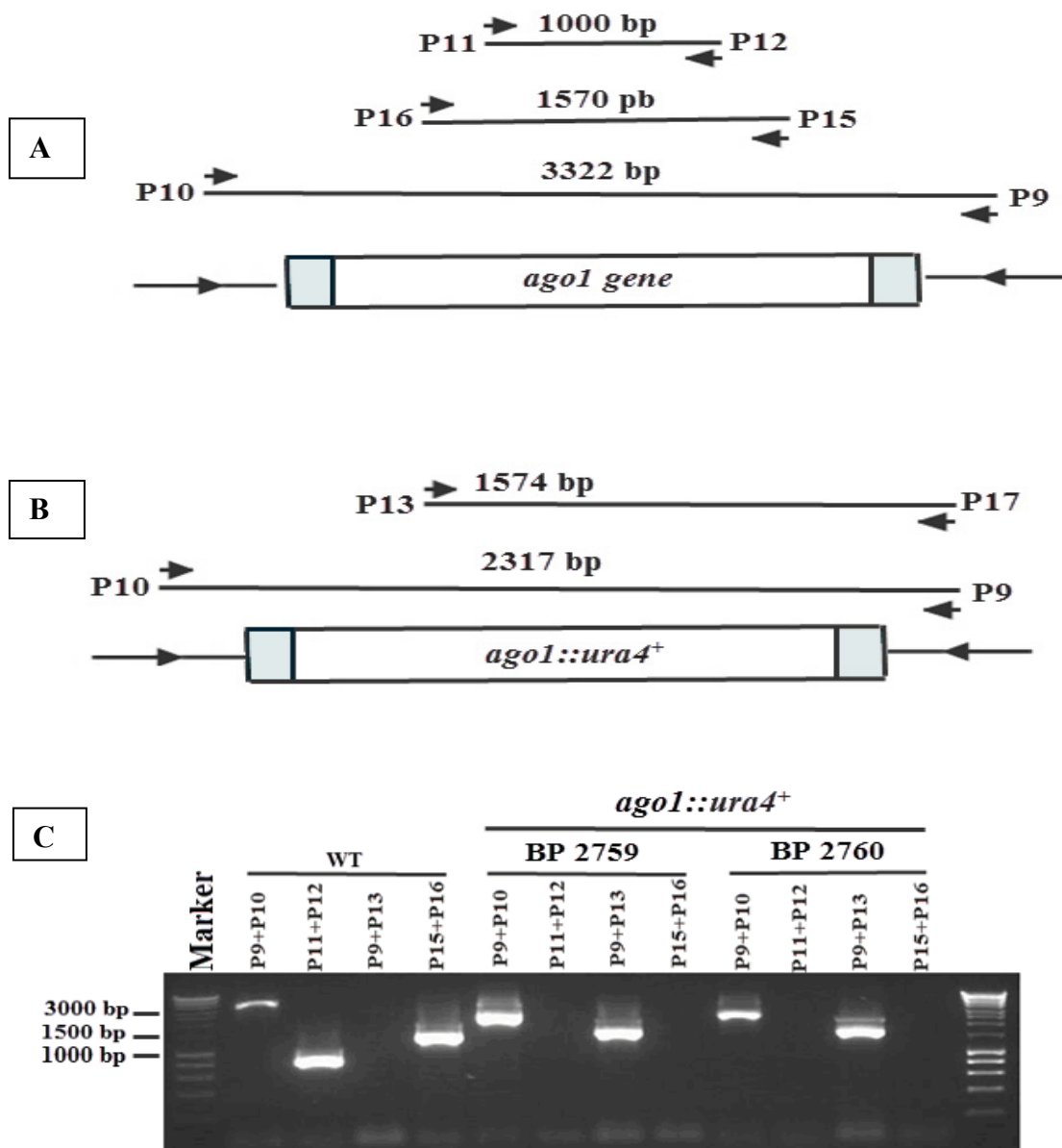


Figure 4.2 PCR results for *ago1Δ* strains.

A. Schematic illustration of the sites of the screening primers used and the size of expected PCR product for wild-type. B. The schematic demonstrates the sites of the screening primers used and the size of expected PCR product for knockout strains. C. DNA from the candidate *ago1Δ* strains subject to a variety of PCR primers where imaging of agarose gel viewing PCR products by using primers from wild-type strain and the *ago1Δ* haploid strains. The expected product size of P 9 and P10 in the wild-type strain is ~3322 bp and ~2317 bp in the knockout. Primers P21-P22 and P11-P12 produce ~1570 bp and 1000 bp in wild-type respectively, and no product was generated in the *ago1Δ* strain, this is further confirmation that *ura4⁺* has replaced the *ago1* in *tsn1Δ* strains.

4.2.2 Microtubule destabiliser sensitivity tests of *tsn1Δ ago1Δ* and *tsn1Δ dcr1Δ* double mutant strains

The centromere is the chromosomal site where kinetochores assembly occurs for spindle attachment and separation of chromosome to daughter cells (Choi *et al.*, 2011; Allshire, 2001). *S. pombe* centromeres are surrounded by inverted arrays of pericentromeric DNA, embedded in heterochromatin which is necessary for their normal function, and so it is required for segregation of chromosomes (Djupedal and Ekwall 2009; Goto and Nakayama, 2012). In *S. pombe* accurate centromeres function requires RNAi to generate histone modification for heterochromatin formation (Zaratiegui *et al.*, 2011; Kamminga *et al.*, 2011). The microtubule-destabilizing drug thiabendazole (TBZ) has antimitotic activity by inhibition of microtubule functions. Cells with defective centromere activity show sensitivity to sub-lethal levels of TBZ. Given that Translin has been implicated in RNAi regulation in *Drosophila* and humans we set out to further explore TBZ sensitivity of *tsn1Δ* / RNAi double mutants. In the previous chapter we tested single *tsn1Δ* mutants for TBZ sensitivity and found no increased TBZ sensitivity. Here we tested double mutant strains (Figure 4.3), where we tested two independently generated double mutant strains for both *ago1Δ* and *dcr1Δ* BP2759, BP2760 (both *ago1Δ tsn1Δ*) and BP2748, BP2749 (both *dcr1Δ tsn1Δ*), and the single mutants BP2758 (*ago1Δ*), BP2747 (*dcr1Δ*), BP1079 (*tsn1Δ*), to determine if the double mutant has any increased sensitivity to sub-lethal levels of TBZ by using spot test assays. Plates were incubated for 3-4 days at 30°C, 33°C, 35°C. Double *dcr1Δ tsn1Δ* mutants strains BP2748, BP2749 were found to be more sensitive to TBZ than either the *dcr1Δ* or *tsn1Δ* single mutant strains. We initially used 15 µg/ml and 12.5 µg/ml TBZ at a range of temperatures (Figures 4.3 and 4.4). 15 µg/ml TBZ was toxic to most of the *dcr1Δ* and *ago1Δ* strains, but use of 12.5 µg/ml appeared to demonstrate that

the *dcr1Δ tsn1Δ* strains were more sensitive to TBZ than the *dcr1Δ* single mutant or the *tsn1Δ* single mutant, which exhibited wild-type resistance to this level of TBZ. This was apparent at all temperatures tested (Figure 4.4). To gain greater resolution of sensitivity differences we reduced the TBZ concentration to 10 μg/ml; at this concentration a clear sensitivity difference of at least 10-fold can be seen between *dcr1Δ tsn1Δ* double mutants and the *dcr1Δ* single mutant (Figure 4.5). Interestingly, the *ago1Δ* exhibits a greater sensitivity to TBZ than the *dcr1Δ* single mutant, and the *ago1Δ tsn1Δ* double mutants do not exhibit increased sensitivity to TBZ relative to the *ago1Δ* single mutant. These data clearly distinguish the relationship between Translin and the different RNAi component genes *ago1Δ* and *dcr1Δ*.

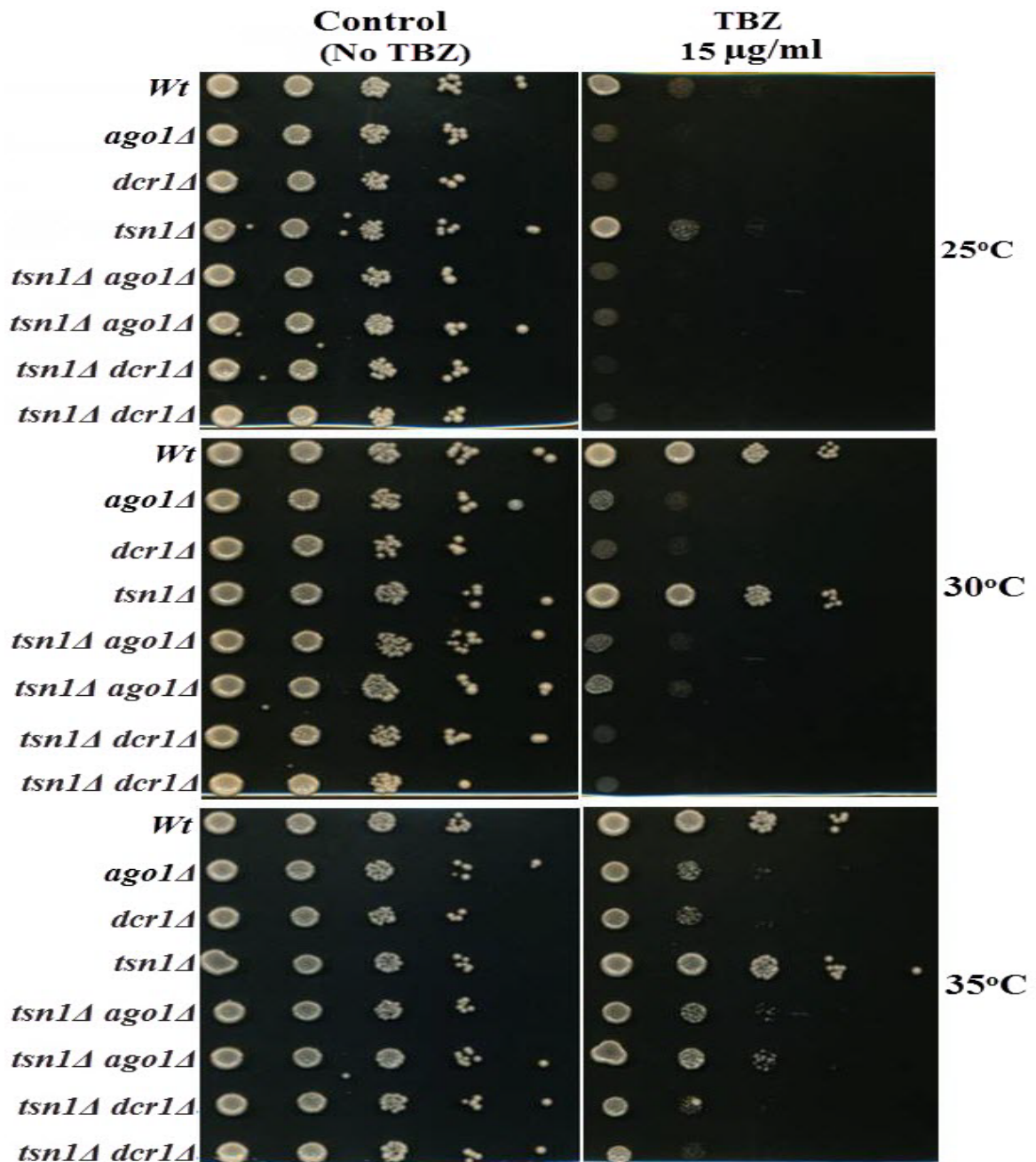


Fig. 4.3 TBZ sensitivity assay using 15 µg/ml TBZ.

Cells were spotted on media contain 15 µg/ml TBZ and incubated at 30°C, 33°C and 35°C for approximately 3 days, where *tsn1Δ* single mutant demonstrate no sensitivity to the microtubule inhibitors thiabendazole (TBZ) at all different temperatures as compared to the control strain. However both *dcr1Δ tsn1Δ* double mutant strains BP2748, BP2749 (*dcr1Δ tsn1Δ*) show more sensitivity to TBZ at all different and temperatures.

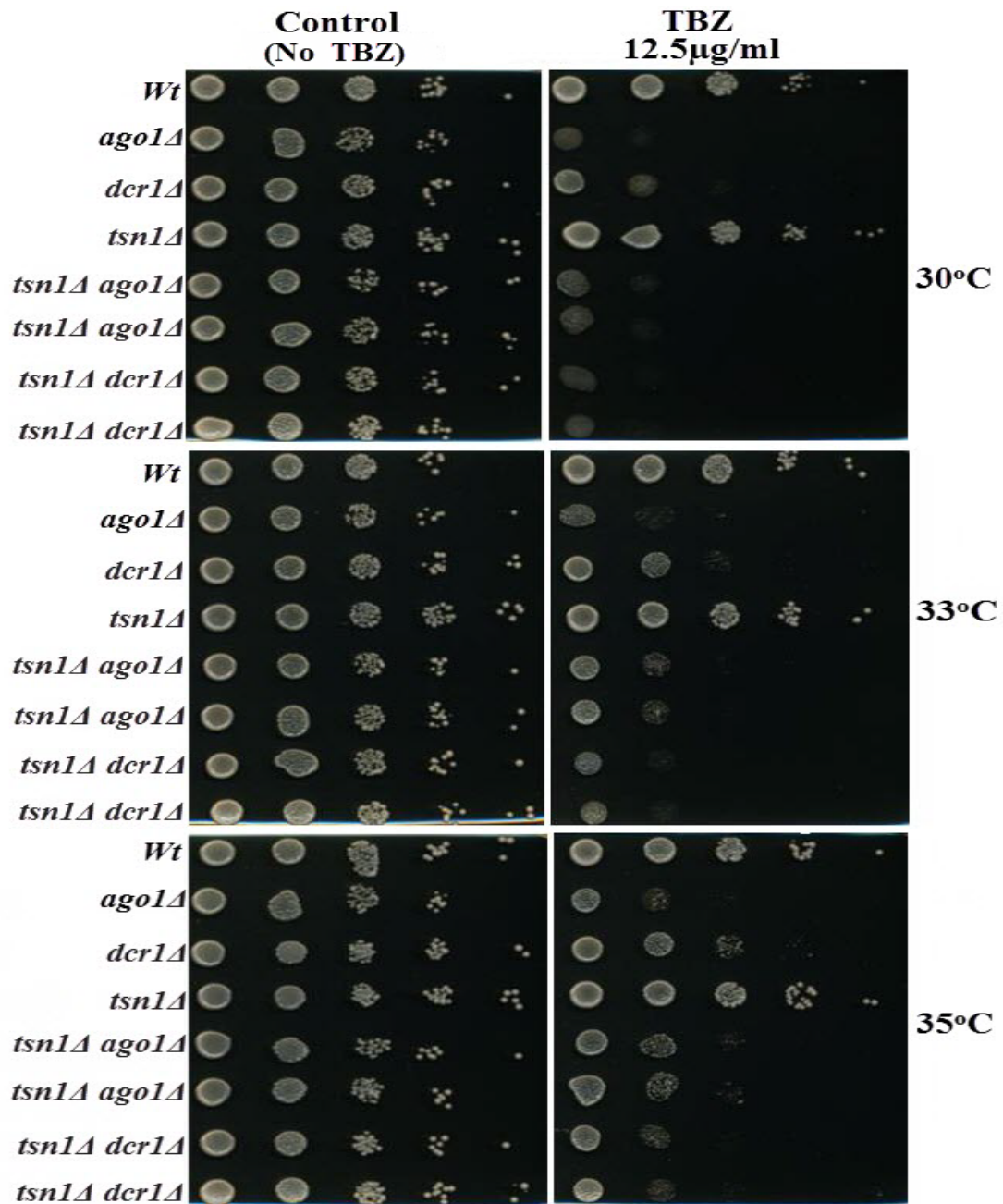


Fig. 4.4 TBZ sensitivity assay using 12.5 µl/ml TBZ.

Cells were spotted on media contain 12.5 µg/ml TBZ and incubated at 30°C, 33°C and 35°C for approximately 3 days, where *tsn1Δ* single mutant demonstrate no sensitivity to the microtubule inhibitors thiabendazole (TBZ) at both *dcr1Δ tsn1Δ* different temperatures as compared to the control strain. However both *tsn1Δ dcr1Δ* double mutant strains BP2748, BP2749 (*dcr1Δ tsn1Δ*) show more sensitivity to TBZ at all different and temperatures.

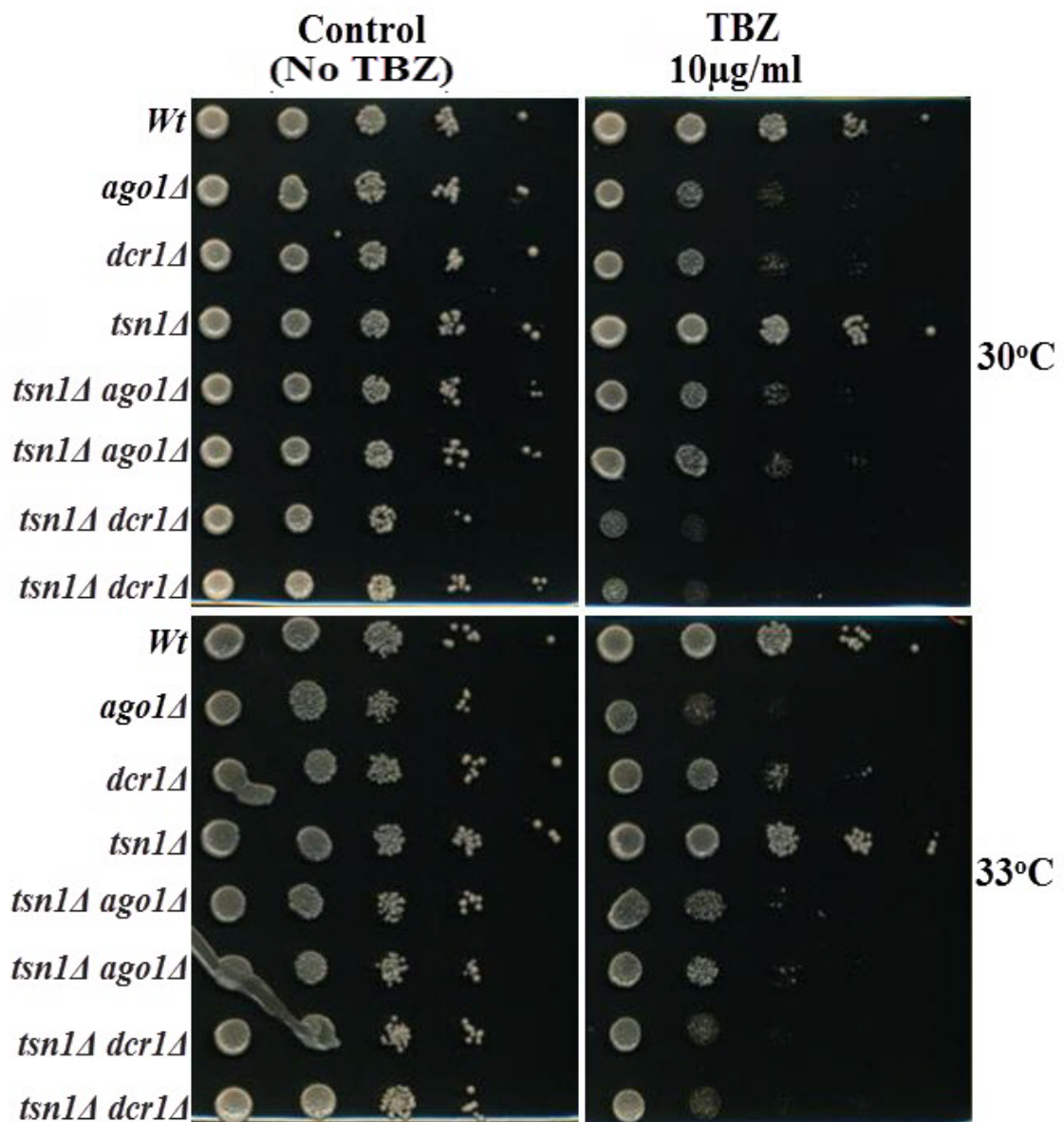


Figure 4.5 TBZ sensitivity assay using 10 µl/ml TBZ.

Cells were spotted on media contain 10 µg/ml TBZ and incubated at 30°C and 33°C and for approximately 3 days, where *tsn1Δ* single mutant demonstrate no sensitivity to the microtubule inhibitors thiabendazole (TBZ) at all different temperatures as compared to the control strain. However both *dcr1Δ tsn1Δ* double mutant strains BP2748, BP2749 (*dcr1Δ tsn1Δ*) show more sensitivity to TBZ at both temperatures.

4.2.3 Minichromosome Loss Assay Experiment

Mitotic chromosome division is a highly precise mechanism and requires a large number of gene product functions for ensuring the preservation of chromosomal euploidy of dividing eukaryotic cells (Javerzat *et al.*, 1996). Segregation of chromosome during mitosis depends upon a number of processes such as the spindle apparatus and the centromere-associated kinetochores (Corbett *et al.*, 2010). Chromosome instability can be readily tested in *S. pombe* using a strain carrying an artificial non-essential minichromosome, which has a high stability in mitotically dividing cells (Javerzat *et al.*, 1996). In *S. pombe* a derivative of chromosome III Chr16 R, carries an *ade6-M216* allele; the full length chromosome III carries an *ade6-M210* allele; *ade6-M210* and *ade6-M216* are complementary and confer prototrophy. When the minichromosome is lost cells lose the *ade6-M216* allele and colonies become red when grown on limiting adenine (as found in YEA) due to a red pigment building up due to the disruption of the adenine biosynthetic way. So Loss of the minichromosome by loss of chromosome segregation function or DNA damage results in visualization of red colonies or white/red sector colonies (Figure 4.6). To determine mini chromosome loss frequencies fluctuation analysis were carried out. All strains were grown to saturation in liquid medium with adenine before being plated, onto low- adenine medium (YEA) and the number of completely red colonies was counted as a measure of cells in the liquid culture which had lost the minichromosome. The number of red colonies was then divided by the total number of colonies to give frequency of minichromosome loss. Median values from 7 independent cultures were obtained; this was repeated three times for each strains and mean values of the medians were plotted. Here, in order to identify whether the single and double deletion strains have increased loss of the mini chromosome we generate strains containing the mini

chromosome and the corresponding *ade6-M210* allele on chromosome III; BP2294 is the wild-type, single knockout strains are BP2421 (*tsn1Δ*), BP2894 (*dcr1Δ*), BP2973(*ago1Δ*), and double mutants strains are BP2977 (*ago1Δ tsn1Δ*) and BP2899 (*dcr1Δ tsn1Δ*). All strains were constructed by direct gene deletion and checked by PCR as previously described. The results demonstrate the *dcr1Δ* and *ago1Δ* single mutant strains have a mini chromosome loss phenotype, as expected. However, as for the TBZ sensitivity assay, the *dcr1Δ tsn1Δ* strain has an elevated mini chromosome instability compared to the *dcr1Δ* or *tsn1Δ* single mutants (Figure 4.7). Again, as with the TBZ sensitivity assay, the *ago1Δ tsn1Δ* exhibited similar level to the *ago1Δ* single mutant.

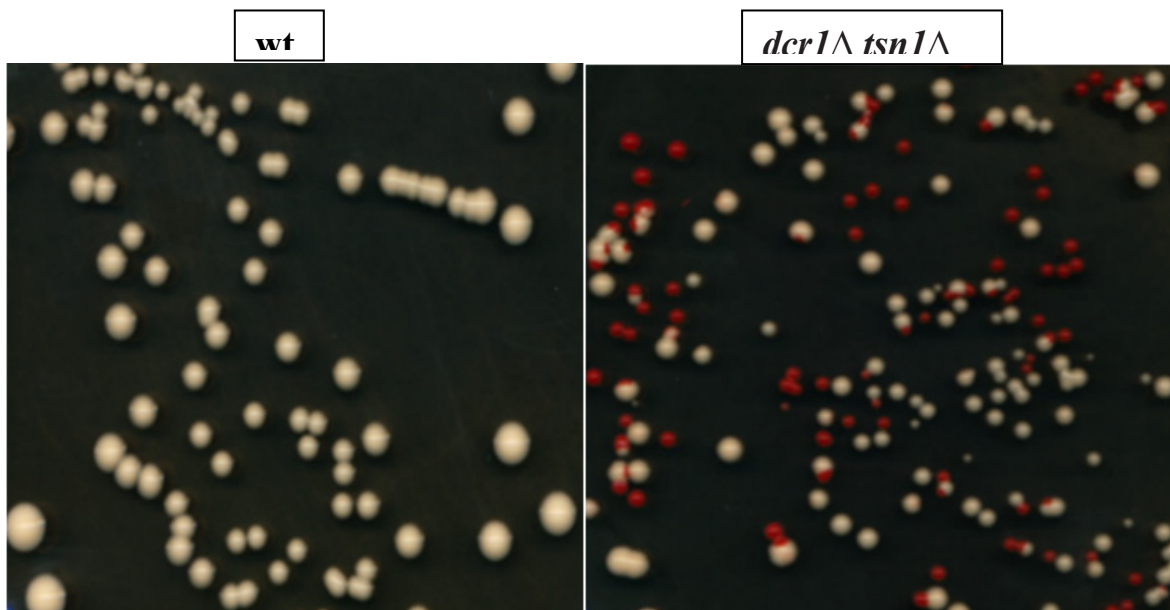


Fig. 4.6 Example of mini chromosome loss phenotype.

Pictures were taken after 4 days of strains growth at 30°C, when the colour was completely developed. BP2294 as wild-type, as the images shows a white colonies. Mini chromosome loss in strains missing (*dcr1Δ tsn1Δ*) result to form red colonies.

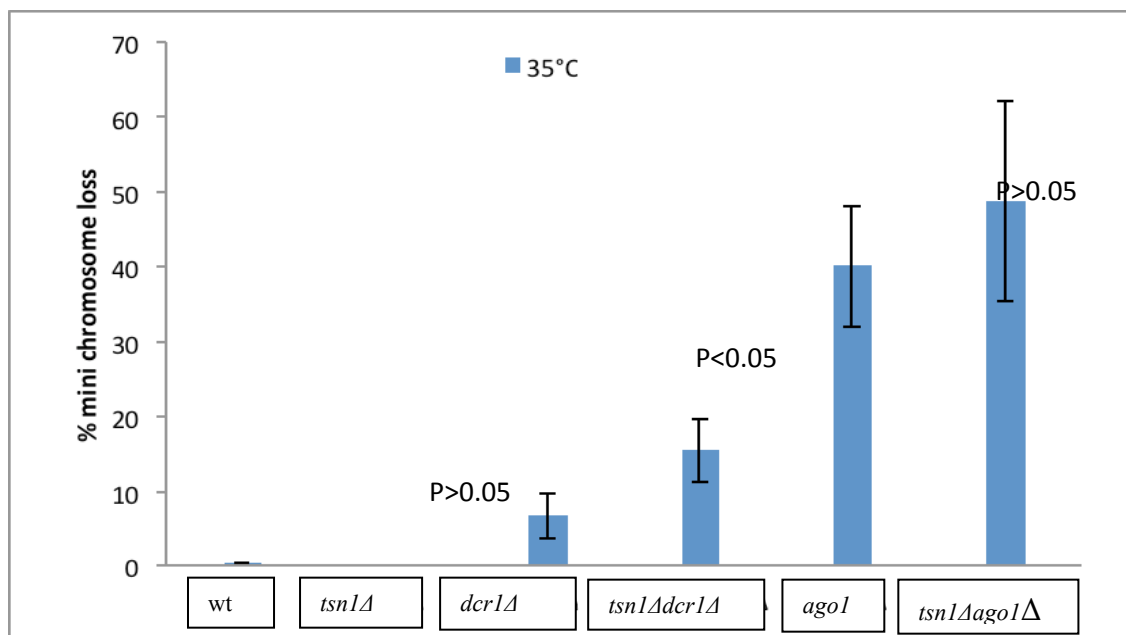
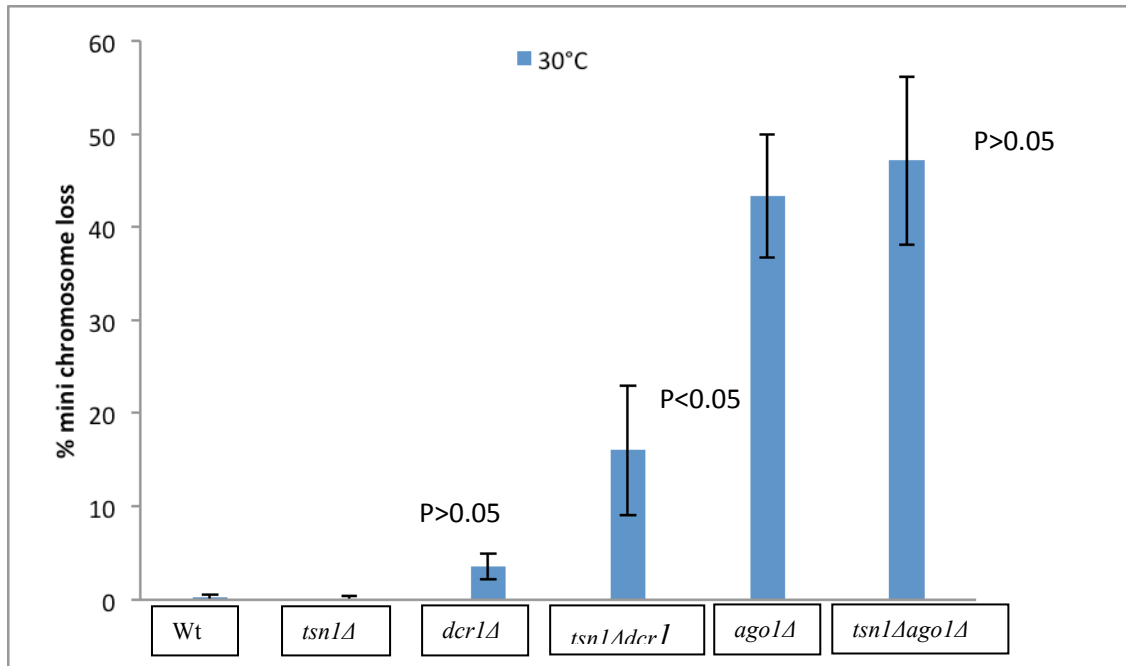


Figure 4.7 Increased mini chromosome loss in *dcr1Δ tsn1Δ* double mutant.

Histograms are mean values of three independently generated median values derived from sets of seven cultures per strain raw data can be found in Appendix I. Cultures were tested at 30°C (top) and 35°C (bottom). There is significant increase in the rate of chromosome loss in *tsn1Δ dcr1Δ* mutants compare to a single *dcr1Δ* at both temperatures. Error bars are standard deviations. *P* values were derived by Student's *t*-test. BP2294 is BP2421 (*tsn1Δ*), BP2894 (*dcr1Δ*), BP2973(*ago1Δ*), BP2977 (*ago1Δ tsn1Δ*) and BP2899 (*dcr1Δ tsn1Δ*).

4.2.4 DNA damage sensitivity tests of *tsn1Δ ago1Δ* and *tsn1Δ dcr1Δ* double mutant strains

Due to increased sensitivity of the *dcr1Δ tsn1Δ* double mutants to TBZ and the increased mini chromosome instability, in conjunction with the fact Translin has been implicated in DNA repair, we set out to test whether the double mutants had greater chromosome instability due to failure to repair DNA damage. We tested for sensitivity to a range of DNA damaging agents, camptothecin which inhibit DNA enzyme topoisomerase I (Fig.4.8), methyl methane sulfonate (MMS) which is considered as an alkylating agent and cancerogenic (Fig 4.9), phleomycin is antibiotic that intercalates DNA (Fig. 4.10), UV irradiation (Fig 4.11), mitomycin C (MMC) is a potent DNA crosslinker (Fig 4.12), which cover a range of types of DNA damage, and the DNA replication inhibitor hydroxyurea (HU) (Fig 4.13). All double mutants lacked significant sensitivity to any of the DNA damaged agents at all the temperatures tested (see Figures 4.8- 4.13). A *rad3Δ* (check point defective) mutant strain was used as a positive control to demonstrate the DNA damaging agents were active.

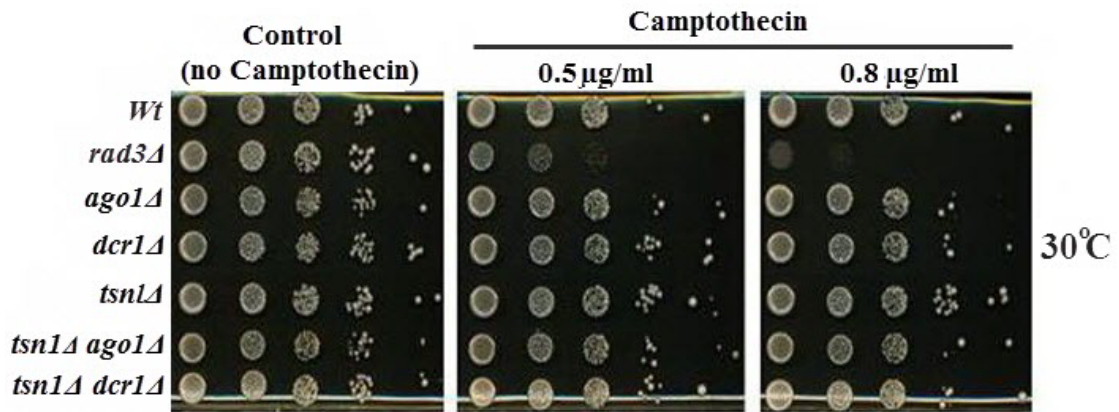


Fig.4.8 Camptothecin spot tests of *tsn1Δ* single and double mutants.

The *tsn1Δ* double mutants strains tested (*ago1Δ tsn1Δ*), (*dcr1Δ tsn1Δ*), demonstrate no increase in sensitivity to CPT compared to wild-type at 30°C. *rad3Δ* cells were used as a positive control.

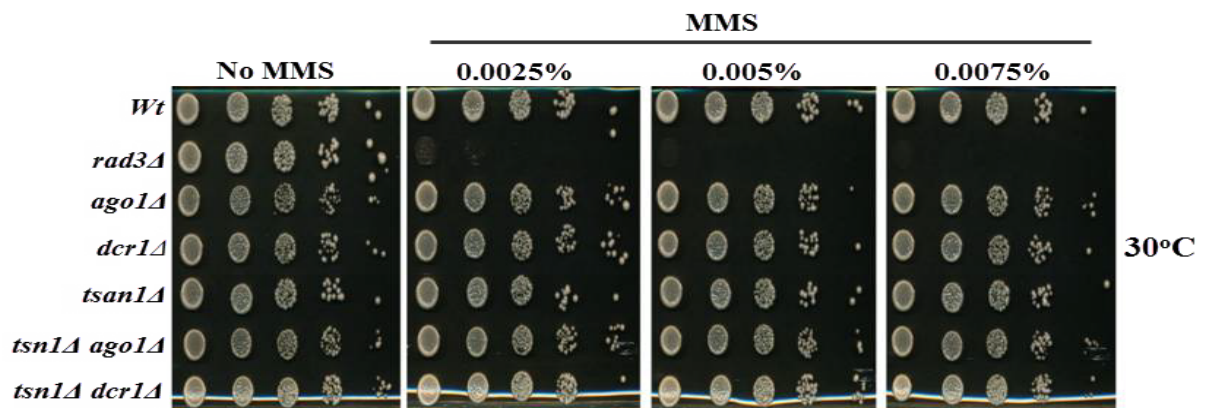


Fig. 4.9 MMS sensitivity test.

Dropped cultures of double mutant (*ago1Δ tsn1Δ*) (*dcr1Δ tsn1Δ*), strains did not show any sensitivity as compared to the wild-type strain to MMS. *rad3Δ* strain used as a positive control.

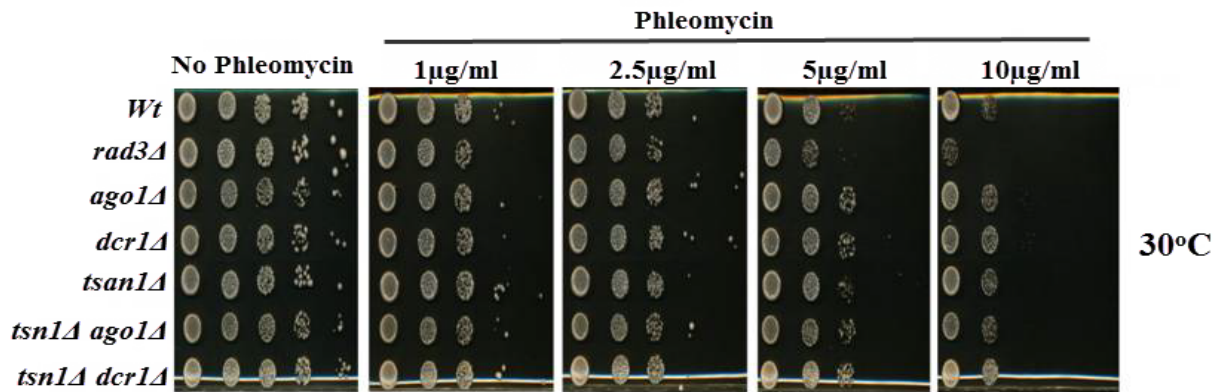


Fig. 4.10 Phleomycin sensitivity test.

Phleomycin spot tests demonstrate that *ago1Δ tsn1Δ*, *dcr1Δ tsn1Δ* double mutants do not exhibit any increased sensitivity to phleomycin compared to wild-type strain. *rad3Δ* strain used as a positive control.

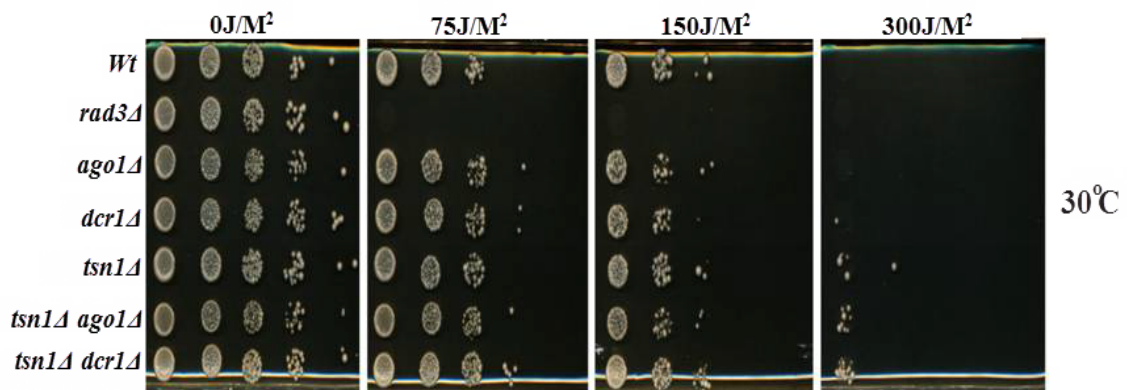


Figure 4.11 UV irradiation sensitivity test.

DNA damage sensitivity tests demonstrate that *ago1Δ tsn1Δ*, *dcr1Δ tsn1Δ* double mutants show no increase in sensitivity to UV relative to wild-type cells. *rad3Δ* strain used as a positive control.

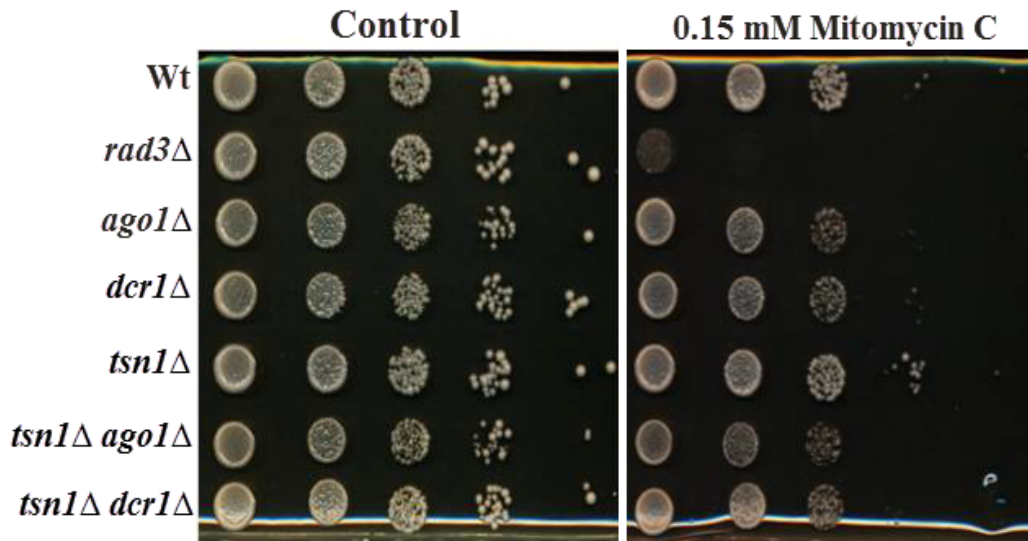


Figure 4.12 Mitomycin C sensitivity test.

Spot tests demonstrate non specific sensitivity to Mitomycin C for *ago1Δ tsn1Δ*, *dcr1Δ tsn1Δ* double mutant strains relative to the wild-type. *rad3Δ* strain used as a positive control.

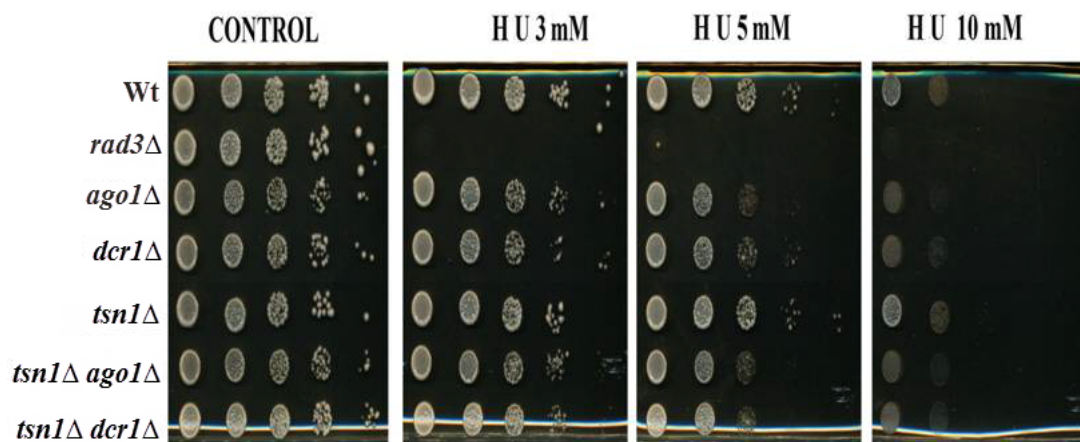


Figure 4.13 HU sensitivity tests.

Spot tests demonstrate non-specific sensitivity to HU for *ago1Δ tsn1Δ*, *dcr1Δ tsn1Δ* double mutants relative to the wild-type. *rad3Δ* strain used as a positive control.

4.3 Discussion

4.3.1 Hypersensitivity to a microtubule poison in double mutant strains (*tsn1Δ dcr1Δ*)

Centromeres are heterochromatic regions required to form the link between the chromosome and the microtubules of the mitotic and meiotic spindle apparatus, integrity of centromeric heterochromatin requires the RNAi machinery (Murakami *et al.*, 2007). Cells with defective centromere activity are normally sensitivity to sub-lethal levels of microtubule destabilizing drugs such as thiabendazole, TBZ (Murakami *et al.*, 2007). Translin may have a role in cell proliferation, as in confocal microscopic studies of the cell cycle in Human Embryonic Kidney (HEK) cells, has demonstrated an enrichment of Translin at the centrosomes and in metaphase it is placed on mitotic spindles and midbodies (Ishida *et al.*, 2002; Yang and Hecht 2004; Jaendling and McFarlane, 2010). Consistent with this, diminution of Translin or Trax by RNAi slows cell division (Yang and Hecht 2004). We examined whether this was also the case for the single mutant *tsn1Δ* and double mutants (*tsn1Δ dcr1Δ*/*tsn1Δ ago1Δ*).

The finding that *tsn1Δdcr1Δ* mutants are more sensitive to TBZ than the *dcr1Δ* single mutant demonstrates that Translin plays a role in cell survival in response to microtubule destabilisation in the absence of dicer. The fact that both independently isolated *tsn1Δdcr1Δ* strains behave in the same fashion indicates that this is a real observation and is unlikely to be due to an aberrant secondary mutation developed during the construction of the double mutant. This is the first observation of a reproducible and significant biological defect attributable to loss of Translin function in *S. pombe*. Moreover, other observations of reported functional defects ascribed to loss of Translin function are subtle, at best, making this the first major functional role identified for Translin.

One possible explanation for this observation is that Translin plays a role in the RNAi pathway which is not essential when dicer is present, but plays a subsidiary role in the absence of dicer, although it cannot completely compensate for loss of dicer as *dcr1Δ* single mutant exhibits significant sensitivity to TBZ. Translin has been reported to be associated with RNase activity and it may be the case that this RNase activity can fulfil a role in a dicer-independent pathway which can maintain heterochromatin function. If loss of Translin in the absence of dicer results in a further defect in heterochromatin function at centromeres, then this would account for the increased sensitivity to TBZ observed here. In the next chapter the question of whether Translin plays a role in centromeric heterochromatin in the absence of dicer is addressed directly.

Interestingly, the increased sensitivity to TBZ is not seen for DNA damaging agents, and so the proposal that loss of Translin could result in general cellular defect due to defects in processing tRNA precursors (Li *et al.*, 2012) does not seem to provide a credible explanation for the functional specificity seen here.

Another compelling observation to come from these studies is that whilst the *tsn1Δdcr1Δ* exhibits increased sensitivity to TBZ relative to the *dcr1Δ* single mutant, the *tsn1Δ ago1Δ* only exhibits the same sensitivity as the *ago1Δ*. This places Translin in the same genetic pathway as argonaute and suggests that an argonaute pathway utilises both Translin and dicer, but is predominantly reliant upon dicer.

4. 3.2. No sensitivity for DNA damage test of *tsn1Δ ago1Δ* and *tsn1Δ dcr1Δ* double mutants.

Translin has been implicated in DNA damage response, where in HeLa cells nuclear Translin levels are increased when these cells are treated with DNA damage agents

Mitomycin C and Cisplatin (Fukuda *et al.*, 2008; Ishida *et al.*, 2002; Yang *et al.*, 2004; Kasai *et al.*, 1997). However, we found that none of our single or double mutants are sensitive to DNA damage. This observation is consistent with previous studies in *Drosophila* embryos which found no increase in sensitive to DNA damage agents in Translin mutants compare to wild-type (Yang *et al.*, 2004; Claußen *et al.*, 2006). All single mutant (*tsn1Δ*) strains and double mutants (*tsn1Δ dcr1Δ*, *tsn1Δ agoΔ*) were tested for sensitivity to camptothecin, methyl methane sulfonate (MMS), phleomycin, UV, mitomycin C, and HU. These strains demonstrated no sensitivity to any of the agents, these observation suggest that Translin does not have a role in DNA damage recovery in the presence or absence of RNAi regulators.

4.3.3. Enhanced chromosomal instability in the absence of dicer and Translin

The mini chromosome loss assays are consistent with the TBZ assays, which indicate that the enhanced sensitivity of the *tsn1Δdcr1Δ* double mutants is related to enhanced genome instability. This instability is not related to decreased ability to cope with DNA damage as we have demonstrated that the double mutants can cope well with a wide range of types DNA damage. Thus it appears from this work that Translin does indeed play a role in maintaining chromosome stability, but only in the absence of dicer. Whether this is due to defective heterochromatin formation and centromere function will be addressed in the following chapter.

4.4 Conclusions

- Translin plays a dicer-independent biological role in maintaining genome stability.
- The role of Translin in maintaining genome stability is not a requirement to mediate the response to DNA damage.
- It remains unknown whether Translin functions to maintain heterochromatin function.

Chapter Five

Analysis of Translin double mutants and centromeric gene silencing

5.1 Introduction

Genomes of unicellular eukaryotes, such as *S. pombe*, have large blocks of heterochromatin, this includes centromeres, telomeres, the silent mating type locus, and the ribosomal DNA (rDNA) (Reyes-Turcu *et al.*, 2012; Noma *et al.*, 2004; Allshire *et al.*, 1994; Huisinga *et al.*, 2009). Marker genes inserted within these regions are repressed and subjected to gene silencing (Huisinga *et al.*, 2009). Heterochromatin has a crucial role in the regulation of transcriptional repression, genomic stability, gene regulation, and segregation of chromosomes during mitotic and meiotic cell proliferation (Trewick *et al.*, 2007; Sugiyama *et al.*, 2005; Ekwall *et al.*, 1999; Steiner *et al.*, 1993). *S. pombe* has three chromosomes each one has a complex centromeric regions of different sizes occupying 38, 65, and 97 kb of DNA (Allshire, 1996). All of these centromeres contain inverted repeated (nontranscribed) DNA sequences of the inner most repeats (*imr*) and heterochromatic outer repeats (*otr*; also termed K repeats) (Huisinga *et al.*, 2009; Smirnova and McFarlane, 2002; Trewick *et al.*, 2007). In the previous chapter it was demonstrated that Translin has a redundant role to dicer in maintaining chromosome stability. Given role of dicer in controlling centromeric heterochromatin it was hypothesised that the increased mini chromosome instability in the *tsn1Δ dcr1Δ* double mutant was due to an increased defectiveness of centromeric heterochromatin/centromeric function. In this chapter this hypothesis is tested by exploring the level of gene silencing in the distinct centromeric domains in *tsn1Δ dcr1Δ* double mutants.

5.2 Results

5.2.1 Strains construction

In previous experiments we construct mutant strains using the *ura4⁺* gene as the selectable marker (see section 4.2), and the antibiotic resistance cassette *kanMX6*. However, in the current experiments use the *ura4⁺* gene as the reporter marker used to measure centromeric gene silencing. Moreover, unlike nutritional markers, antibiotic markers are not likely to have an effect on the growth rate, thus *dcr1Δ* and *ago1Δ* null mutant constructions were based on the *natMX6*, nourseothricin resistance system described in Bähler *et al.* (1998), Gene deletion primers were designed that had 120 bp of homology to the sequence immediately upstream and downstream of the *dcr1* and *ago1* open reading frame (ORF) and 20 bp of homology to the *pFA6a* plasmid which contained the *natMX6* marker gene (Hentges *et al.*, 2005). Candidate strains were selected by nourseothricin resistance and correct gene deletion were confirmed PCR (Figure 5.1 and 5.2).

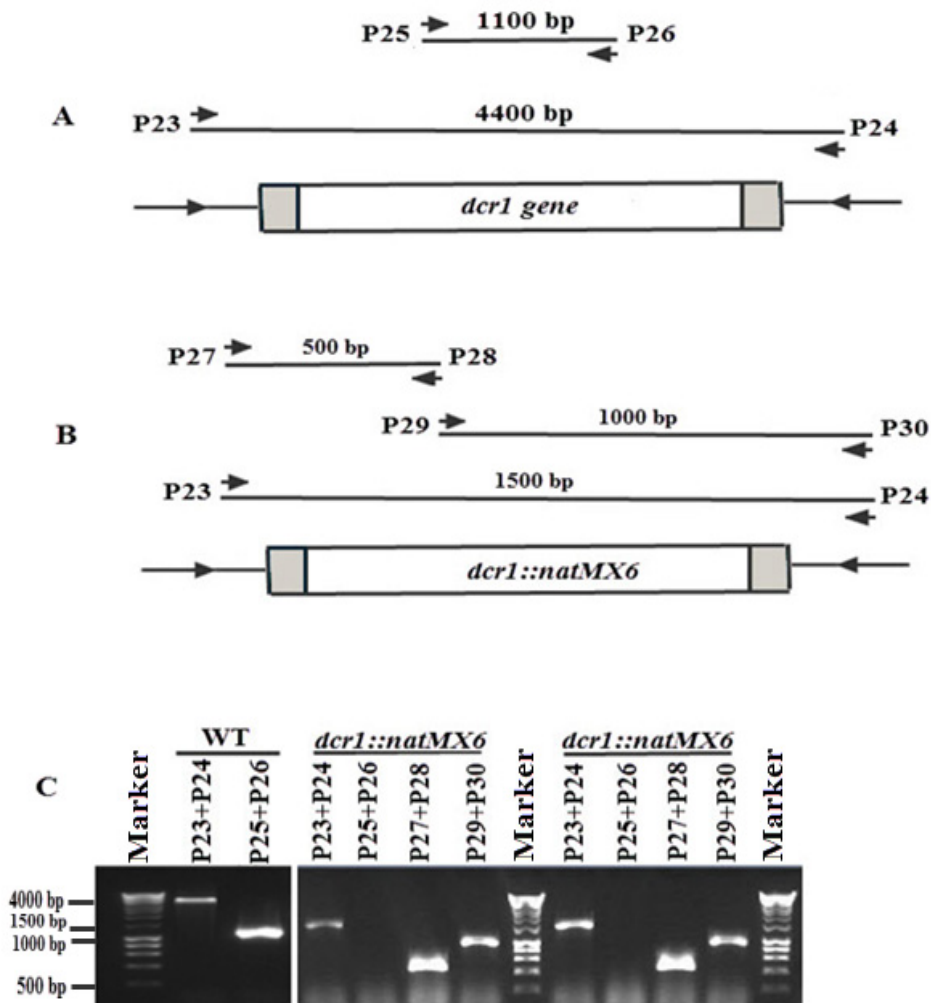


Figure 5.1 PCR bands for *dcr1Δ* strain construction.

A. Schematic illustration of the sites of the screening primers used and the size of expected PCR product for wild-type. B. The schematic demonstrates the sites of the screening primers used and the size of expected PCR product for knockout strains by using *natMX6* cassette. C. DNA from candidate *dcr1Δ* strains subject to a variety of PCR primers where imaging of agarose gel viewing PCR products by using primers P23 and P24 from wild-type strain and the *dcr1Δ* haploid strains. The expected product size of P23 and P24 for the wild-type strain is ~4400 bp and ~1500 bp for the knockout. Primers P27-P28 and P29-P30 produce ~500 bp and ~1000 bp for the knockout respectively, and no product was generated in the *dcr1Δ* strain for primers P25-P26 while in wild-type is ~1100 bp, this is further confirmation that *natMX6* has replaced the *dcr1* gene in *tsn1Δ* strains.

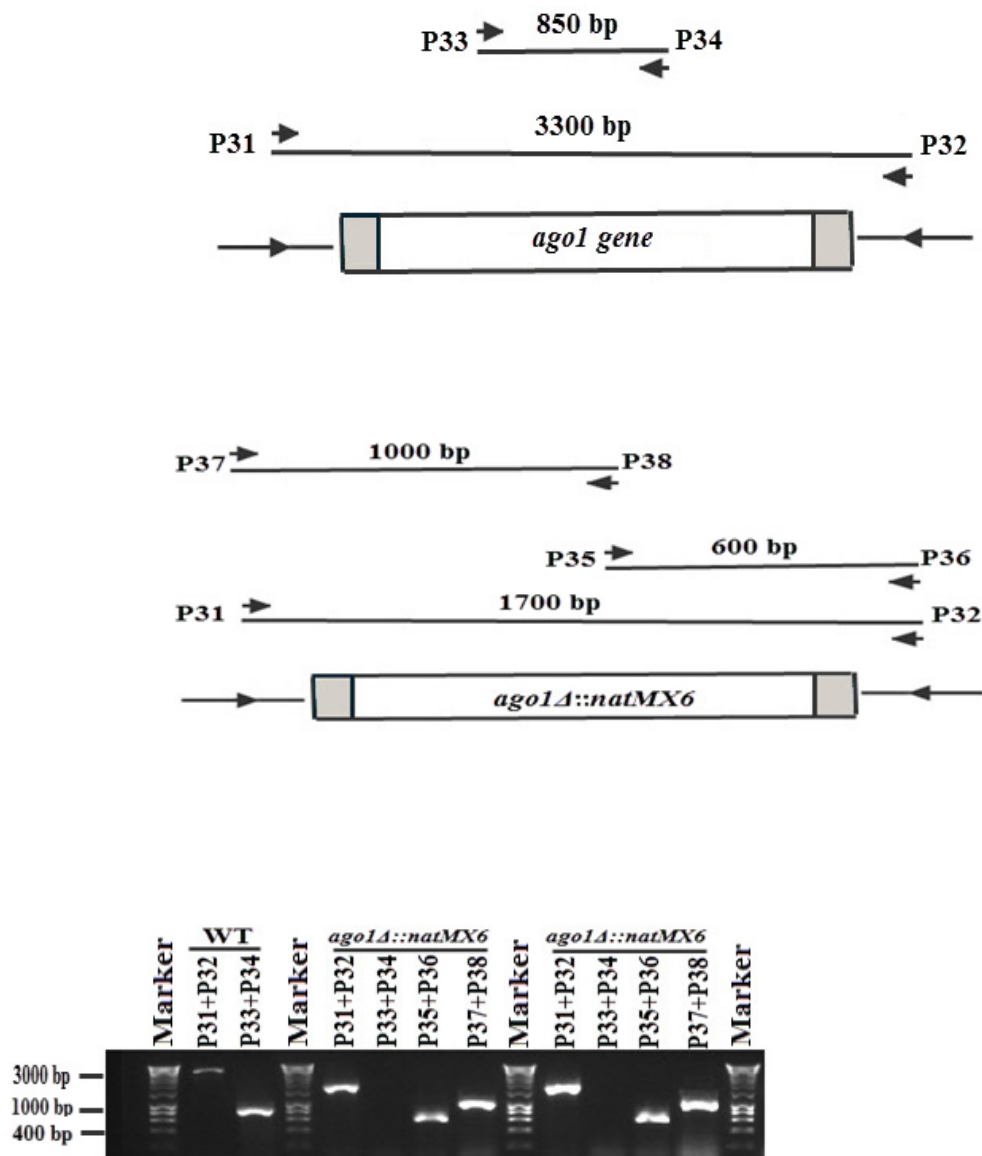


Figure 5.2 PCR bands for *ago1Δ* strain construction.

A. Schematic illustration of the sites of the screening primers used and the size of expected PCR product for wild-type. B. The schematic demonstrates the sites of the screening primers used and the size of expected PCR product for knockout strains by using *natMX6* cassette .C. DNA from the candidate *ago1Δ* strains subject to a variety of PCR primers where imaging of agarose gel viewing PCR products by using primers P31 and P32 from wild-type strain and the *ago1Δ* haploid strains. The expected product size of P31 and P32 for the wild-type strain is ~3300 bp and ~1700 bp for the knockout. Primers P35-P36 and P37-P38 produce ~600 bp and ~1000 bp in knockout respectively, and no product was generated in the *ago1Δ* strain for primers P33-P34 while in wild-type is ~850 bp, this is further confirmation that *natMX6* has replaced the *ago1Δ* in *tsn1Δ* strains.

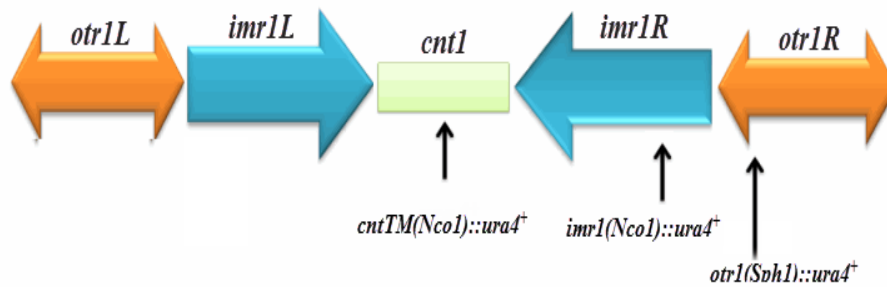


Figure 5.3 The Schematic demonstrates centromere 1.

It consists of a central core (*cnt*) of nonrepetitive sequence flanked by innermost repeats (*imr*) and outer repeats (*otr*) placing of marker *ura4⁺* genes wherever in the centromere results in their transcriptional repression (Adapted from Ekwall *et al.*, 1999; Pidoux and Allshire, 2005).

5.2.2. Analysis of centromeric gene silencing in double mutants

Centromeres play a crucial function in chromosome separation (Rudd *et al.*, 2003). Whereas it is a particular chromosomal section that play as a place for protein–DNA and protein–protein connections to make the kinetochore construction and for accurate chromosome separation (Howman *et al.*, 200; Goshima *et al.*, 1999). Several human cancer demonstrate abnormal chromosome segregation, as result of deregulation of centromeric integrity, these changes reflect deregulation of the centromeres function (Frescas *et al.*, 2008; Manning *et al.*, 2010). Consistently, in *S. pombe* the RNAi pathway is necessary for the construction of pericentric heterochromatin and accurate chromosome separation (Reddy *et al.*, 2011). For functional analysis of centromeres, marker genes that are artificially inserted into the *S. pombe* centromeric heterochromatic regions has allowed the identification of mutations which interrupt the structure of this repressed chromatin and allowing expression of marker gene (Volpe *et al.* , 2003). Therefore, transcriptionally silent due to RNAi-mediated heterochromatin and strains with *ura4*⁺ deleted at the endogenous locus carrying the centromeric silenced *ura4*⁺ gene can grow on 5-FOA but they become sensitive to 5-FOA, a drug which is toxic to *ura4*⁺ cells when heterochromatin-mediated gene silencing becomes deregulated and centromeric *ura4*⁺ is expressed. The *ura4*⁺ gene has been introduced into the three heterochromatic sub regions of centromere I (*cenI*) the *cnt1*, *imr1* and *otr1* in distinct strains (refer to Fig. 5.3). To test whether a double mutant of *tsn1*Δ *dcr1* Δ or *tsn1*Δ and *ago1* Δ alleviates heterochromatin-mediated silencing in centromeres carrying the *ura4*⁺ marker located in the three sub regions of the centromeres were generated. Saturated cultures from a series of five-fold dilutions were spotted onto YEA, supplemented EMM, EMM lacking uracil to assess the low levels of *ura4*⁺ expression and 500 μg/ml 5-FOA plates to evaluate expression of *ura4*⁺ genes form centromere. Sensitivity to 5-FOA was tested at a range of temperatures (30°C, 33°C, 35°C).

5.2.2.i. *cnt1::ura4*⁺

At all temperature the Translin single mutant exhibited full suppression of the *ura4*⁺ marker gene indicating heterochromatic silencing was fully functional. In both *ago1Δ* and *dcr1Δ* single mutants silencing was deregulated, but no additional defect was apparent in the double mutant (Figure 5.4).

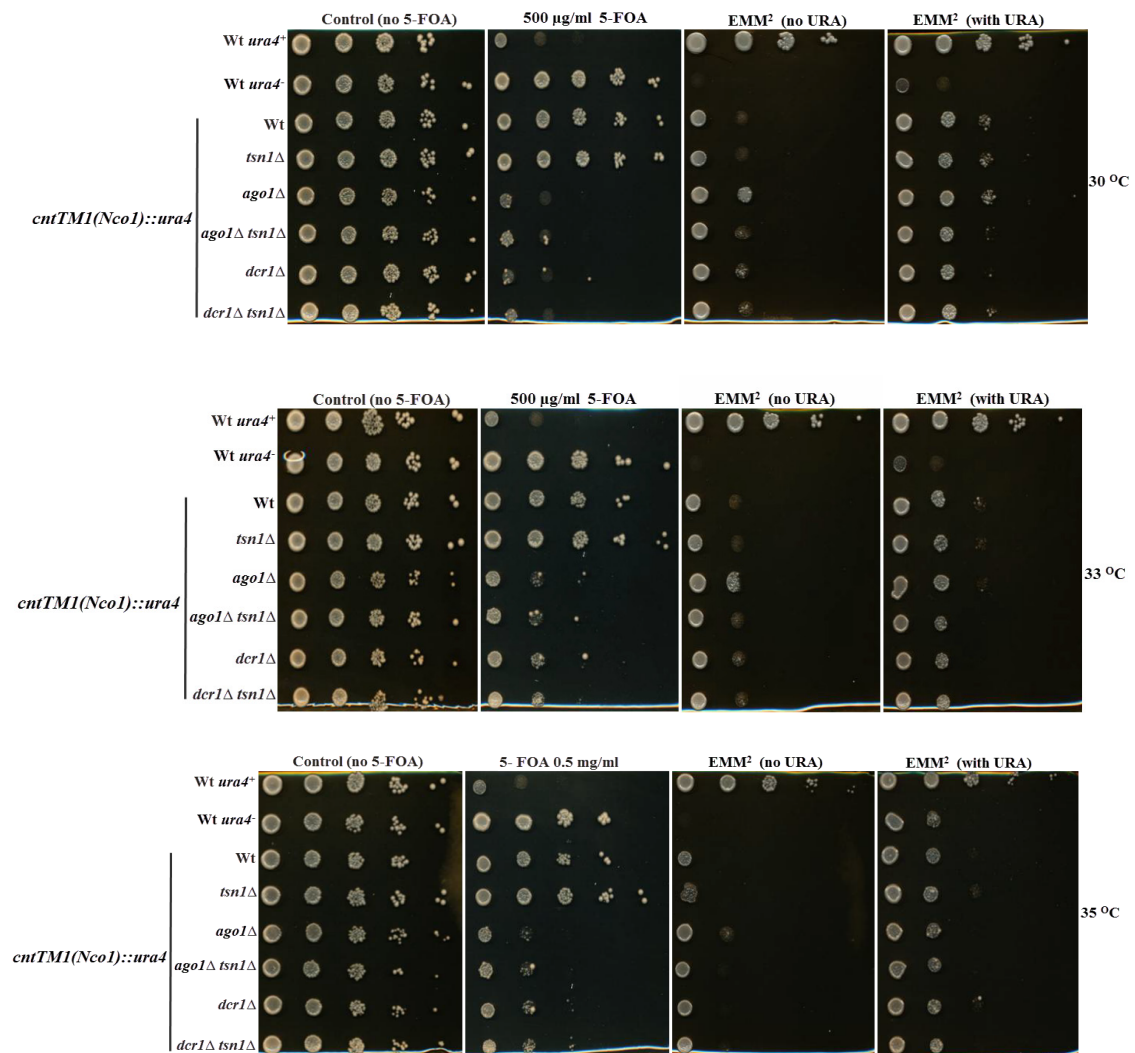


Figure 5.4 5-FOA experiment *cnt* double mutants.

wt BP1, isogenic wt BP90, parents mutants strains BP2203, BP2418 (*tsn1Δ*), BP3001 (*ago1Δ*), BP3103 (*dcr1Δ*), BP 2989 (*dcr1 Δ, tsn1Δ*), BP 3111 (*tsn1Δ, ago1Δ*), were spotted on YEA media and plates containing 500 µg/ml 5-FOA, and minimal media where the ura concentration was 200mg/L. Growth of null mutants at 30°C, 33°C, 35°C for approximately 3 days. Growth tested of (*dcr1 Δ tsn1Δ*) and (*tsn1Δ ago1Δ*), do not appear more sensitive to (5-FOA).

5.2.2.ii. *imr1::ura4*⁺

At all temperature the Translin single mutant exhibited full suppression of the *ura4*⁺ marker gene indicating heterochromatic silencing was fully functional. In both *ago1Δ* and *dcr1Δ* single mutants silencing was deregulated, but no additional defect was apparent in the double mutant (Figure 5.5).

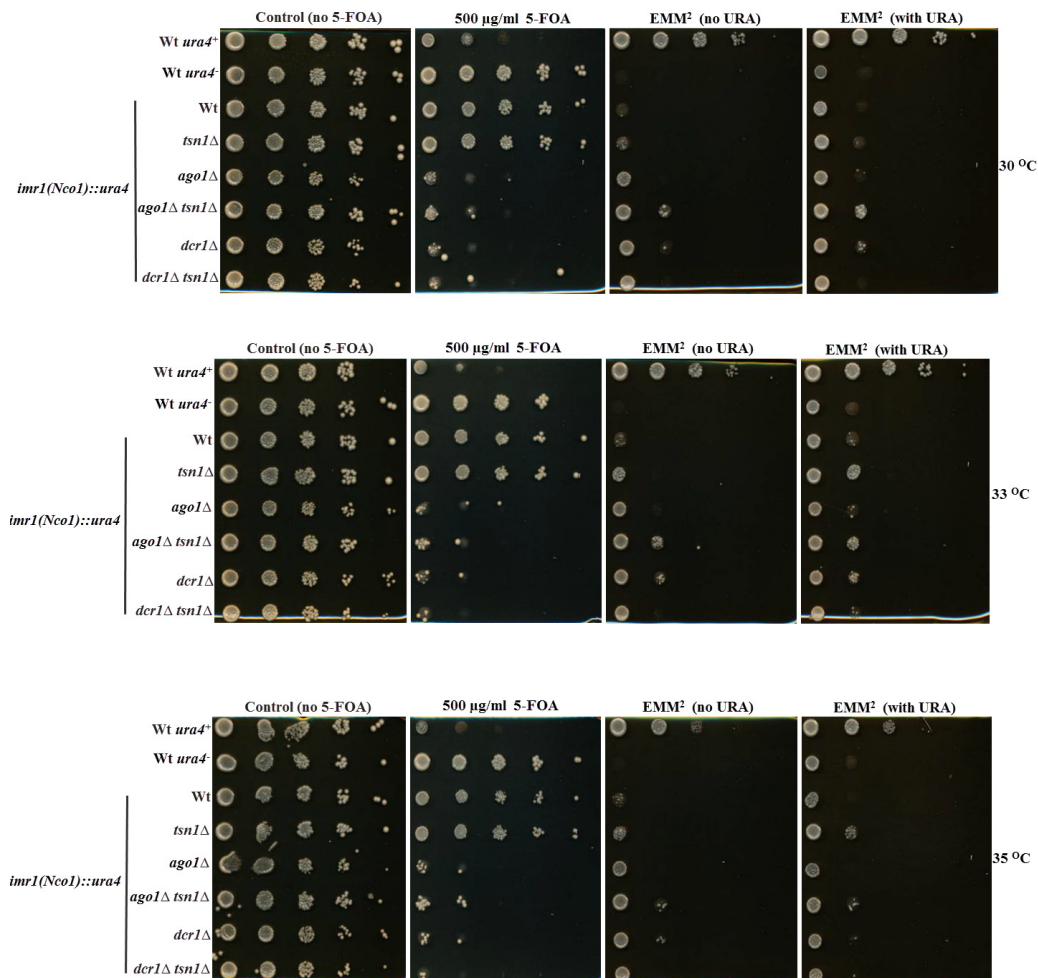


Figure 5.5 5-FOA Experiment *imr* double mutants.

wt BP1, isogenic wt BP90, parents mutants strains BP2506, BP2500 (*tsn1Δ*), BP3005 (*ago1Δ*), BP3028 *dcr1 Δ*, BP 3026 (*dcr1 Δ, tsn1Δ*), BP3003 (*tsn1Δ ago1Δ*), were spotted in serial dilution on YEA and plates containing 500 ug/ml 5-FOA, and minimal media. Growth of single and double mutants at, 30°C, 33°C, 35°C for approximately 3 days. Growth tested of (*dcr1 Δ tsn1Δ*) and (*tsn1Δ ago1Δ*), do not appear more sensitive to 5-FOA.

5.2.2.iii. *otr1::ura4*⁺

At all temperature the Translin single mutant exhibited full suppression of the *ura4*⁺ marker gene indicating heterochromatic silencing was fully functional. In both *ago1Δ* and *dcr1Δ* single mutants silencing was deregulated, but no additional defect was apparent in the double mutant (Figure 5.6).

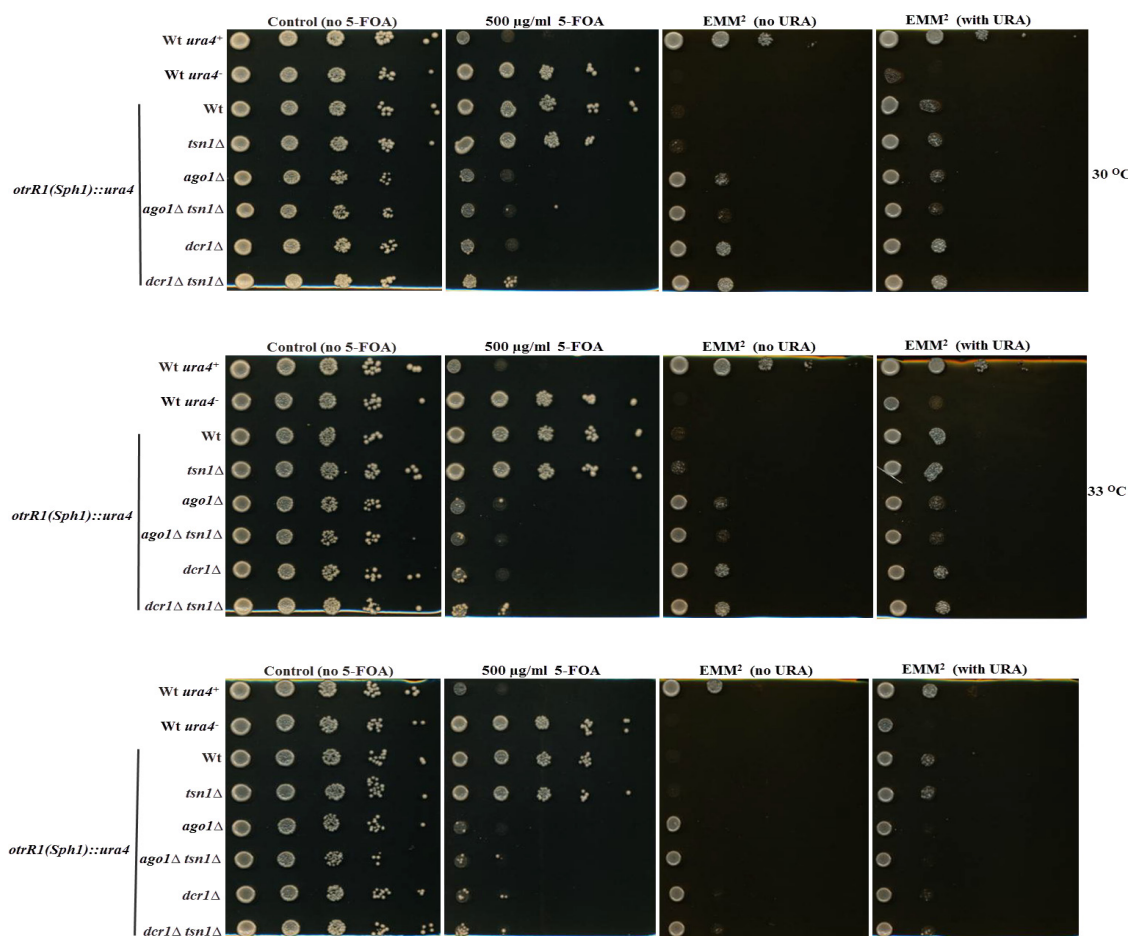


Figure 5.6 5-FOA Experiment *otr* double mutants .

Whole single and double mutants were tested for growth at three different temperature, 30°C, 33°C, 35°C for approximately 3 days. wt BP1, isogenic wt BP90, parents mutants strains BP2204, BP2420 (*tsn1Δ*), BP3109 (*ago1Δ*), BP2985 *dcr1 Δ*, BP 3024 (*dcr1 Δ tsn1Δ*), BP2995 (*tsn1Δ ago1Δ*), were spotted in serial dilution on YEA and plates containing 500 ug/ml 5-FOA, and minimal media. Growth tested of (*dcr1 Δ tsn1Δ*) and (*tsn1Δ ago1Δ*), do not appear more sensitive to 5-FOA.

5.3 Discussion

Centromere of *S. pombe* is composed of large inverted repetitive elements and two discrete repressed domains that are together necessary for centromere function. These regions are transcriptionally silent and insertion of marker genes results in transcriptional repression of the markers gene (Pidoux and Allshire, 2005; Partridge *et al.*, 2002). This feature of centromeres silencing is frequently used as a genetic means to measure centromeres functionality. Given that we found the *tsn1Δ dcr1Δ* double mutant to exhibit a greater sensitivity to TBZ and enhanced mini chromosome instability (Chapter 4), in combination with a possible link to RNAi function, we predicted that this was caused by a secondary pathway in centromere heterochromatin maintenance. Surprisingly, no additional defect in silencing at *cnt1*, *imr1*, or *otr1* regions was observed. This suggests that there is no measurable additional defect in the centromeres in the double mutants, particularly *tsn1Δ dcr1Δ*. Whilst this means of measuring centromere function might have missed a subtle additional defect, we believe to be unlikely as the additional TBZ sensitivity and chromosome instability were marked.

This finding leads to the speculation that other regions of genomic heterochromatin, such as the telomeres, the rDNA locus and/or the mating-type developmental locus might be influenced by loss of Translin function in the absence of dicer activity. This hypothesis is tested in the next chapter, but the lack of additional centromeric silencing defects for centromere 1 (*cen1*) further indicates that the defects caused by loss of Translin function in the absence of dicer are not having a generalised negative influence on genome activity as suggested by Li and co-workers (2012) who recently found a role for Translin in tRNA processing and proposed that this resulted in defect in a broad range of biological

functions. Indeed, we postulated a highly specific function for Translin for regulating chromosome stability in the absence of dicer; what this might be remains to be elucidated.

5.4 Conclusion

Translin double mutants demonstrate no loss of gene silencing function

Chapter Six

Affect of Translin double mutants in gene silencing at rDNA, *mat* locus and subtelomeric heterochromatin

6.1 Introduction

Centromeres are not the only a place of *S. pombe* chromosomes that are heterochromatic, mating loci (*mat*), telomeres, and rDNA repeats also consist of heterochromatin and transcriptionally silent regions (Michael *et al.*, 2011; Hansen, *et al.*, 2011; Talbert and Henikoff, 2006).

In mammals, in addition to *S. pombe*, the end of linear chromosomes consists of repetitive tandem elements and stabilised by specialised DNA-protein structures known as telomeres, which appear cytological compressed (heterochromatin) where the gene adjacent to it is transcriptionally repressed because distribution of silent heterochromatin (Bah, and Azzalin, 2012). This phenomenon, known as 'telomeric silencing' is found from yeast to human. Therefore repressed genes placed nearby telomeres give an excellent model to test heterochromatin role (Smith *et al.*, 2008). In addition, the subtelomeric regions which is enrich for repetitive elements are also heterochromatic and genes placed into these places are also transcriptionally repressed, a phenomenon called telomere position effect (Blasco, 2007; Allshire *et al.*, 1995; Ekwall *et al.*, 1995; Bah, and Azzalin, 2012).

S. pombe is unicellular eukaryotes has a simple life cycle comprise haploid and diploid phase (Kelly *et al.*, 1988). One type of the cell cycle is meiosis which is a vital process in produce of haploid gametes, which is important step for production of genetically individual cells (Krapp *et al.*, 2010; Shigehisa *et al.*, 2010). Genes in cells act in response to different environmental changes, such as changes in temperature and starvation (Krapp

et al., 2010). Meiosis in *S. pombe* produces four spores and is initiated under nitrogen starvation condition (Figure 6.1), which activates genes necessary for sexual development, there are more than one hundred genes activated (Mizuki *et al.*, 2011). *S. pombe* naturally grows as haploid cells that has three mating-types genes at the *mat* locus divided into three cassettes; two of them are silent, *mat2P* (h^+) and *mat3M* (h^-), and the third, *mat1*, is active (refer to Fig. 3.8) (Arcangioli and Lahondès, 2000). The spread of heterochromatin in these sites includes the mating loci genes *mat2P* (h^+) and *mat3M* (h^-) and the 11-kb region between them *cenH* repeat that is 96% similar to the outer repeats of centromeres (*dg* and *dh* centromeric repeats) and produces siRNAs and non-coding RNAs (Hansen *et al.*, 2011; Trewick *et al.*, 2007; Talbert and Henikoff, 2006; Noma *et al.*, 2004). The mating-type locus elements are targeted by RNAi to recruit chromatin modifiers and establish silent heterochromatin by guiding non-coding RNAs which have the ability to direct the RNAi factors to somehow facilitate the H3K9me establishment (Bayne *et al.*, 2010). The *mat* locus is located on the right section of chromosome II (Arcangioli and Lahondès, 2000). Furthermore, *mat1* can contain either *mat2P* (h^+) and *mat3M* (h^-) type allele, genetic information which is also stored in silenced form at *mat2P* (h^+) and *mat3M* (h^-), the size of DNA across *mat2-P*, *mat3-M* is about 20 kb, and the intervening area are heterochromatic (Hansen *et al.*, 2011; Klar 2007). In case of switching of mating-type genetic information of a silent loci *mat2P* or *mat3M*, which act as donors are transferred to the active locus, *mat1* (Hansen *et al.*, 2011), by a process of genetic recombination, where the flanking regions of the three mating-type loci are identical, so the recombination is initiated by a break at one of these flanks, and resolved to the other homologous flank (Egel, 2005) and thus determines the mating-type of a cell (Arcangioli and Lahondès, 2000). Consequently, h^+ mating-type locus cells produce P pheromones and make M pheromone receptors for the opposite pheromone on the cell surface and versa vice (Klar, 2007; Nielsen, 1993). In

the presence of an appropriate mating partner the opposite kinds of haploid cells will conjugate to form a zygote following meiosis.

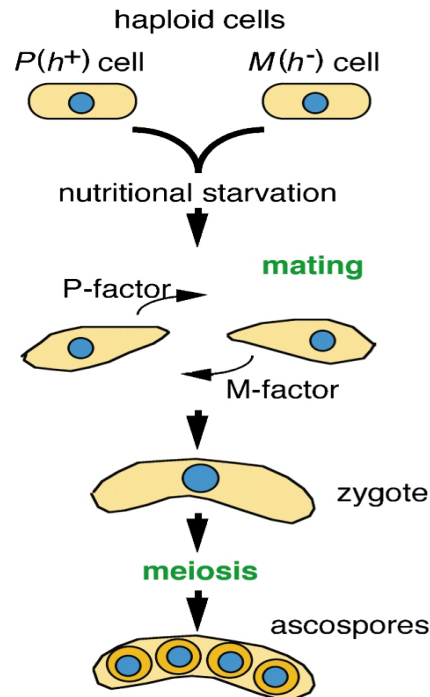


Figure 6.1 Diagram showing the process of mating and meiosis in *S. pombe*.

In case of nutritional reduction, haploid cells of the h^+ and h^- mating type arrest vegetative growth and initiate mating. Cells of the P and M mating-types are communicated by secretion mating pheromone to the opposite mating type cells and conjugate to produce a sporulation (adapted from Yamamoto, 2010).

S. pombe shows one of the two kinds a homothallic or heterothallic life cycle. Under nitrogen starvation conditions, homothallic (h^{90}) cells have the ability to switch their mating type through cell proliferation between plus and minus once time per cell division. Heterothallic cell populations exist as h^+ or h^- cells where cells do not have the ability to switch mating type, therefore population of haploid cells need opposite mating types to generate cell fusion and diploid zygote (Matia-Gonzalez *et al.*, 2012; Kitamura *et al.*, 2001; Klar 2007).

An additional heterochromatic region is the highly repetitive rDNA locus, where many tandemly repeated *rRNA* genes are set within a heterochromatic structure. The role of the heterochromatin is thought to regulate rRNA gene expression and suppress unwanted inter-repeat recombination events (for example see Krings and Bastia, 2004; Huang, 2002).

Here we explore whether Translin plays a redundant role in regulation of heterochromatin-mediated silencing in order to address whether loss of this function might account for the increased chromosome instability observed in *tsn1Δ dcr1Δ* double mutants.

6.2 Results

6.2.1 Examination of gene silencing at the *rDNA* locus in double mutants.

rDNA in most eukaryotic cells which encode ribosomal RNAs are clustered as families of long tandem head-to-tail repeats (Coulon *et al.*, 2004; Kobayashi *et al.*, 2004). As result of the heterochromatic nature of this locus, marker genes placed in the rDNA array are repressed by heterochromatin (Dubey, 2009 and Vasiljeva *et al.*, 2008; Cam *et al.*, 2005). To investigate whether of any single mutant BP2720 (*tsn1Δ*) or double mutants BP3189 (*tsn1Δ dcr1Δ*), BP3193 (*tsn1Δ ago1Δ*) were defective in rDNA silencing a strain the with *ura4⁺* marker gene located in the rDNA arrays (*rDNA::ura4⁺*) was used as a base strain for generating the required single and double mutants, which were generated and checked as previously described(see previous chapters).

Only the required strains were created and generated, the degree of *rDNA::ura4⁺* gene silencing was assayed by testing sensitivity to the drug 5-FOA, which kills cells expressing *ura4⁺*. As can be seen in figure 6.2 loss of the function of any of the RNAi genes seems to have little influence on gene silencing of *rDNA::ura4⁺*. There is certainly no increase of sensitivity to 5-FOA for the *tsn1Δ dcr1Δ* double mutants relative to either single mutant.

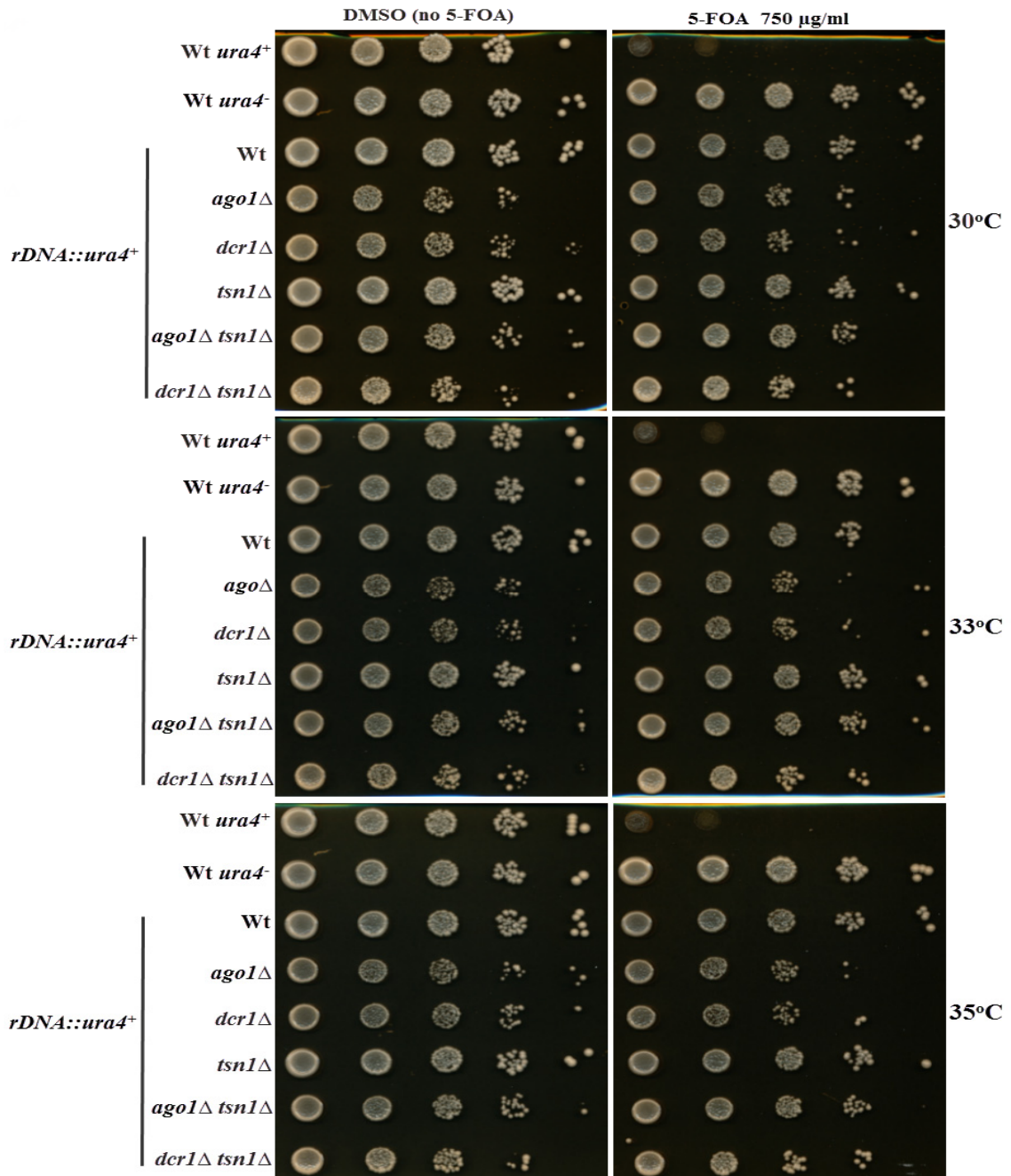


Figure 6.3 Investigation of *rDNA* gene silencing in double (*tsn1Δ dcr1Δ*), (*tsn1Δ ago1Δ*) mutants.

The double mutations did not significantly influence expression of *rDNA::ura4* in comparison to wild-type cells. The cells were tested at 30°C, 33°C and 35°C.

6.2.2 Examination of *mat* locus gene silencing in double mutants.

Starvation induces single cells of opposite mating type of *S. pombe* to mate with one another to produce spores, this switching occurs by transpositions of DNA by a recombination between the silent loci *mat2* or *mat3*, into *mat1* where *mat1* that stored either the P or M mating type allele (Thon and Klar, 1992). The mating-type locus elements are heterochromatic which is distinguished by H3K9me, the existence of chromodomain proteins, and hypoacetylation and targeted by the RNAi machinery (Hansen *et al.*, 2011; Bayne *et al.*, 2010; Vengrova and Dalgaard, 2004; Talbert and Henikoff, 2006). In *S. pombe* the mating-type locus gives a well-defined structure to examine how heterochromatic histone modifications influence gene expression (Hansen *et al.*, 2011). We tested whether single BP2724 (*tsn1Δ*) or double mutants BP3190 (*tsn1Δ dcr1Δ*), BP3195 (*tsn1Δ ago1Δ*) has an influence on mating-type silencing, to do this we used a strain background with the *ura4⁺* marker inserted into heterochromatic *mat* regions in which it is silent. Mutant strains were constructed by PCR previously described. However, the data shows no difference in the *mat* locus silencing between the mutants strains and wild-type strains (Figure 6.3).

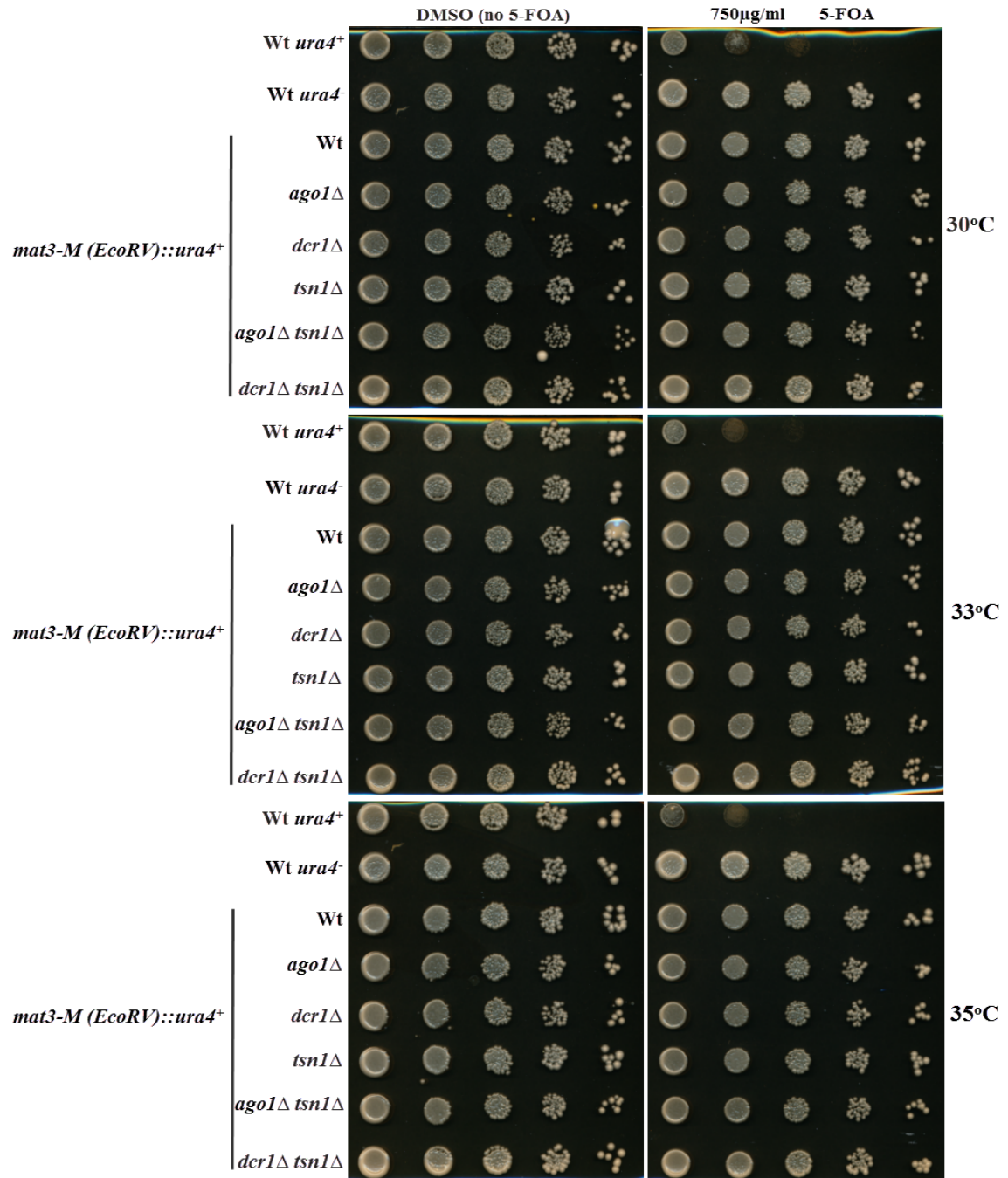
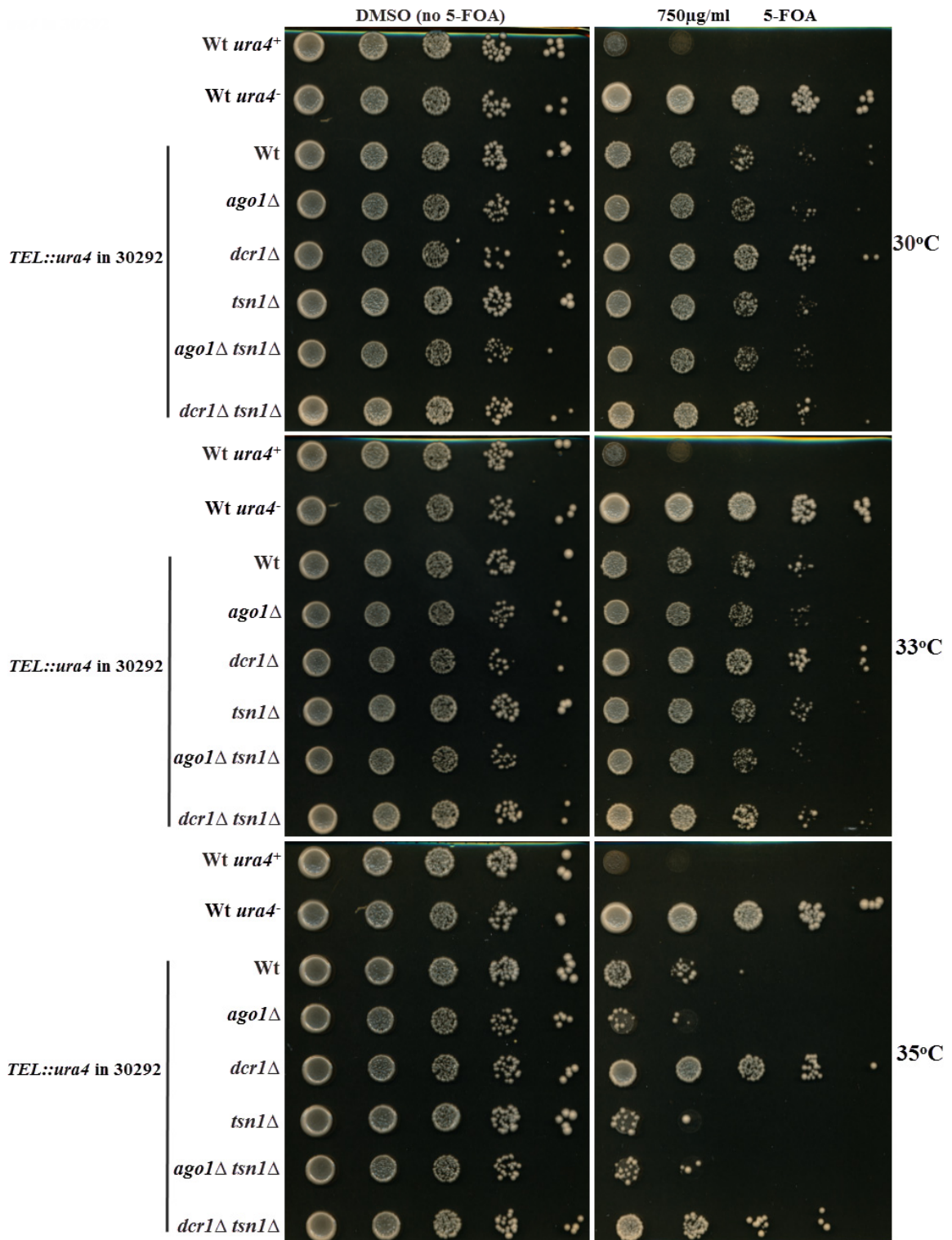


Figure 6.4 Investigation of *mat* loci silencing in double (*tsn1*Δ *dcr1*Δ), (*tsn1*Δ *ago1*Δ) mutants.

The results demonstrate that the double mutations did not significantly influence expression of *ura4*⁺ in the *mat* locus in comparison to wild-type cells. The cells were tested at 30°C, 33°C and 35°C.

6.2.4 Examination of silencing gene in subtelomeric heterochromatin in a double mutants

Telomeres are tandem repeats of short and variation sequences of DNA which protect the ends of linear chromosomes and preserve genome stability (Poon and Mekhail, 2012). Telomeres regions are heterochromatic and genes inserted near telomeres become transcriptionally inactive (Blasco, 2007; Allshire *et al.*, 1995; Ekwall *et al.*, 1995; Miyoshi *et al.*, 2003; Bah and Azzalin, 2012). Kanoh and co-workers (2005) demonstrated that the heterochromatin protein Swi6 (HP1) can establish heterochromatin in *S. pombe* subtelomeric regions independently of the RNAi machinery. They found this to be dependent on the telomere-binding protein Taz1. They determined this RNAi-independence by testing *dcr1Δ* strains, but not *ago1Δ*. They tested gene silencing at various positions away from the telomeres by inserting *ura4⁺* at distinct sites different distance from the telomeres in different strains. We obtained strains in which *ura4⁺* had been inserted approximately 7 kbp from the telomeres (*TEL::ura4⁺-7421*) and approximately 30 kbp from the telomeres (*TEL::ura4⁺-30292*). We generated the appropriate *dcr1Δ/ ago1Δ tsn1Δ* and double mutant combination for these strains and confirmed them as previous described (see previous). We then tested levels of gene silencing at a range of temperatures at both subtelomeric loci. The sub-telomeric 7 kbp position exhibited no measurable loss of silencing in all strains (figure 6.4). At the 30 kbp position the double mutants exhibited no additional loss of silencing relative to at least one of the single mutants at all temperatures using a range of 5-FOA concentrations (Figures 6.5). However, strikingly, the *dcr1Δ* single mutant exhibited a greater degree of silencing than either the *dcr1⁺*, wild-type control or the *ago1Δ* mutant. Whilst this does not provide any indication relating to the function of Translin, it is a remarkable result with respect to sub-telomeric silencing regulation and the role of the RNAi machinery.



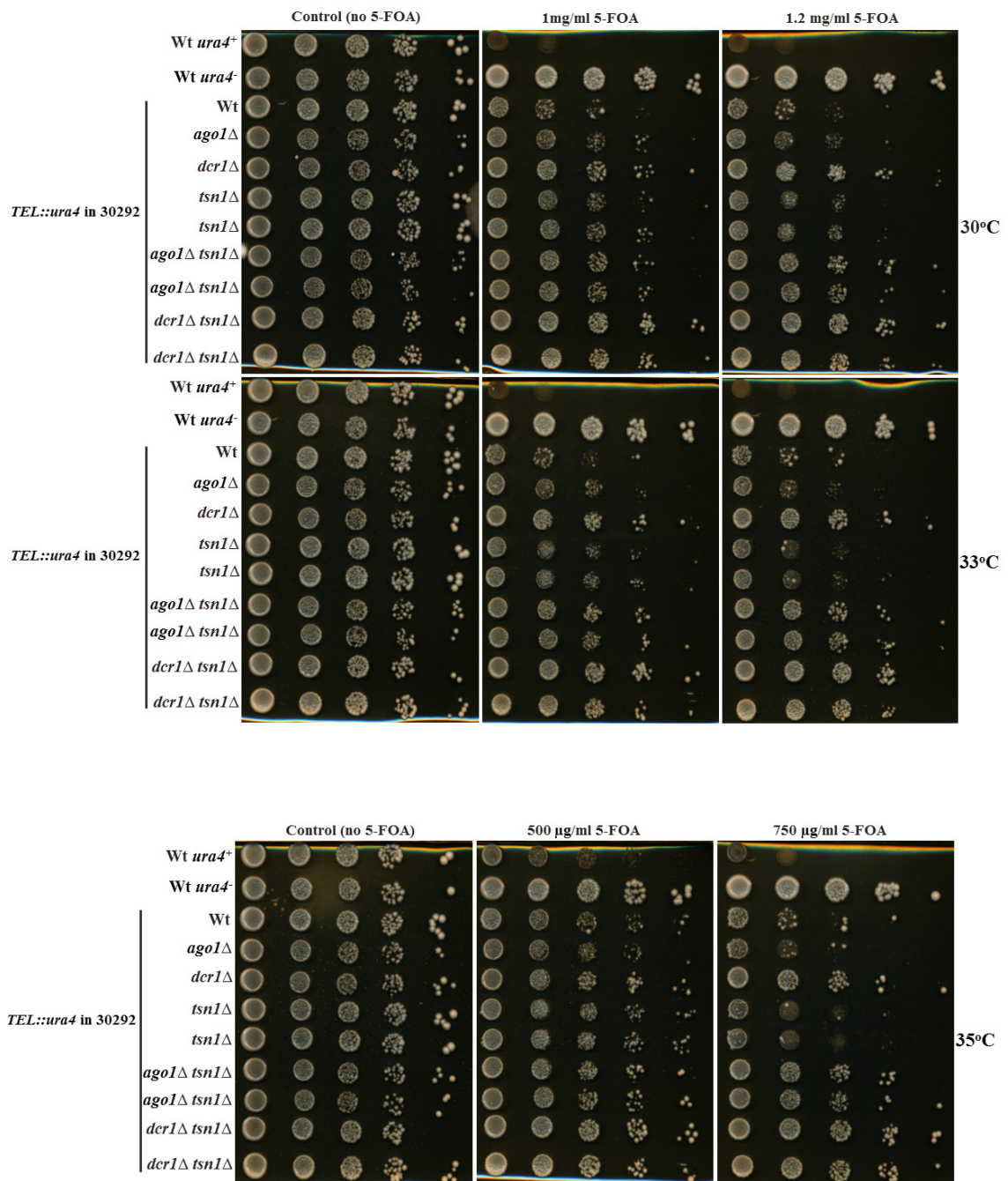


Fig. 6.5 5-FOA sensitivity test of the 30 kbp silencing using 500 µg/ml, 750 µg/ml, 1 mg/ml, 1.2 mg/ml 5-FOA.

Cells were spotted on media contain 1 mg/ml, 1.2 mg/ml 5-FOA and incubated at 30°C, 33°C, and on media contain 500 µg/ml, 750 µg/ml and incubated at 35°C for approximately 3 days.

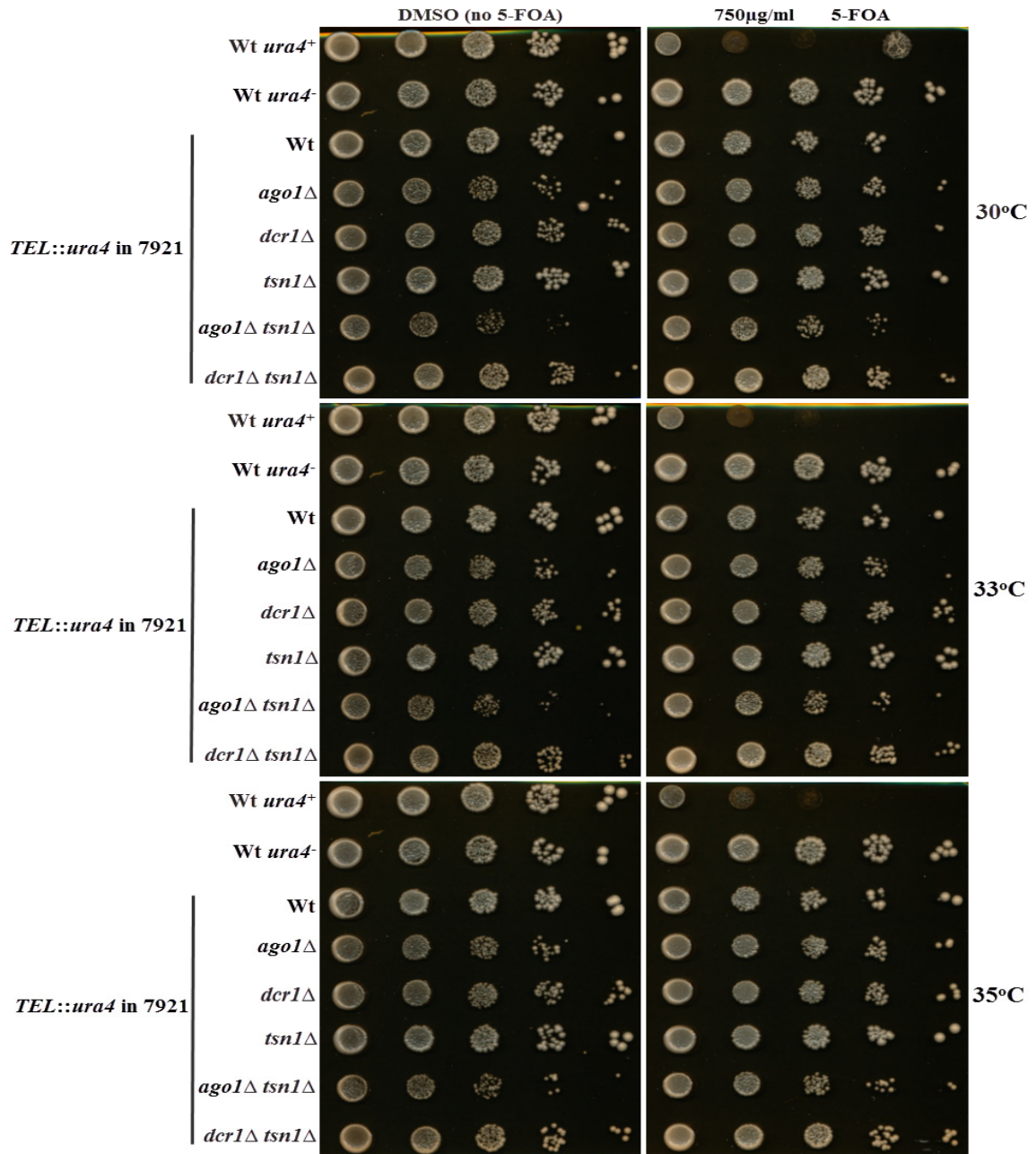


Fig. 6.6 Analysis of gene silencing at the subtelomeric 7 kbp position.

Cells were spotted on media contain 750 µg /ml 5-FOA and incubated at 30°C, 33°C and 35 °C, for approximately 3 days, Double mutants *tsn1*Δ, *ago1* Δ/ *dcr1* Δ double mutant strains show no sensitive on 5-FOA media at all temperatures.

6.2.3 Mating type test in *tsn1Δ*

S. pombe meiosis occurs when the cells exposed to nutrient starvation, particularly in the case of nitrogen diminution where two haploid cells of opposite mating type mate conjugate to form diploid which go through sporulation, (Krapp *et al.*, 2010; Yamamoto, 2010; Dalgaard and Klar, 2000). In *S. pombe* the wild-type h^{90} strains can switch between h^+ and h^- which happens about once in each three cell divisions (Yamamoto *et al.*, 1997). In the sporulation state the cells produce a starch-like compound which can be stained with iodine vapours, whereas inadequately switching strains stain sparingly; so, the effectiveness of switching can be assayed by iodine staining, where the spores stained extremely black when exposure with iodine vapours indicating highly levels of mating and spore formation (Shankaranarayana *et al.*, 2003; Thon and Klar. 1993). In this experiment we test effect of a *tsn1Δ* mutation on mating-type switching competence in the h^{90} background. Mating-type switching levels were tested by the iodine vapour staining assay. The h^{90} *tsn1Δ* strain required for this was generated and tested as previous described. As can be seen from figure 6.6 the *tsn1Δ* h^{90} strain appear to switch *mat* cassettes with an efficiency indistinguishable from the wild-type. Additionally, *S. pombe* mating results in asci with four equally sizes spores (Figure 6.1). If there is a defect in switching at *mat* or a meiotic defect the numbers of asci will be reduced; moreover, some meiotic defects can result in spores/asci with aberrant morphologies. We examined the morphology of spores/asci from *tsn1Δ* h^{90} mating and stained the spore DNA and be found the mutant to indistinguishable from wild-type (Figure 6.7).

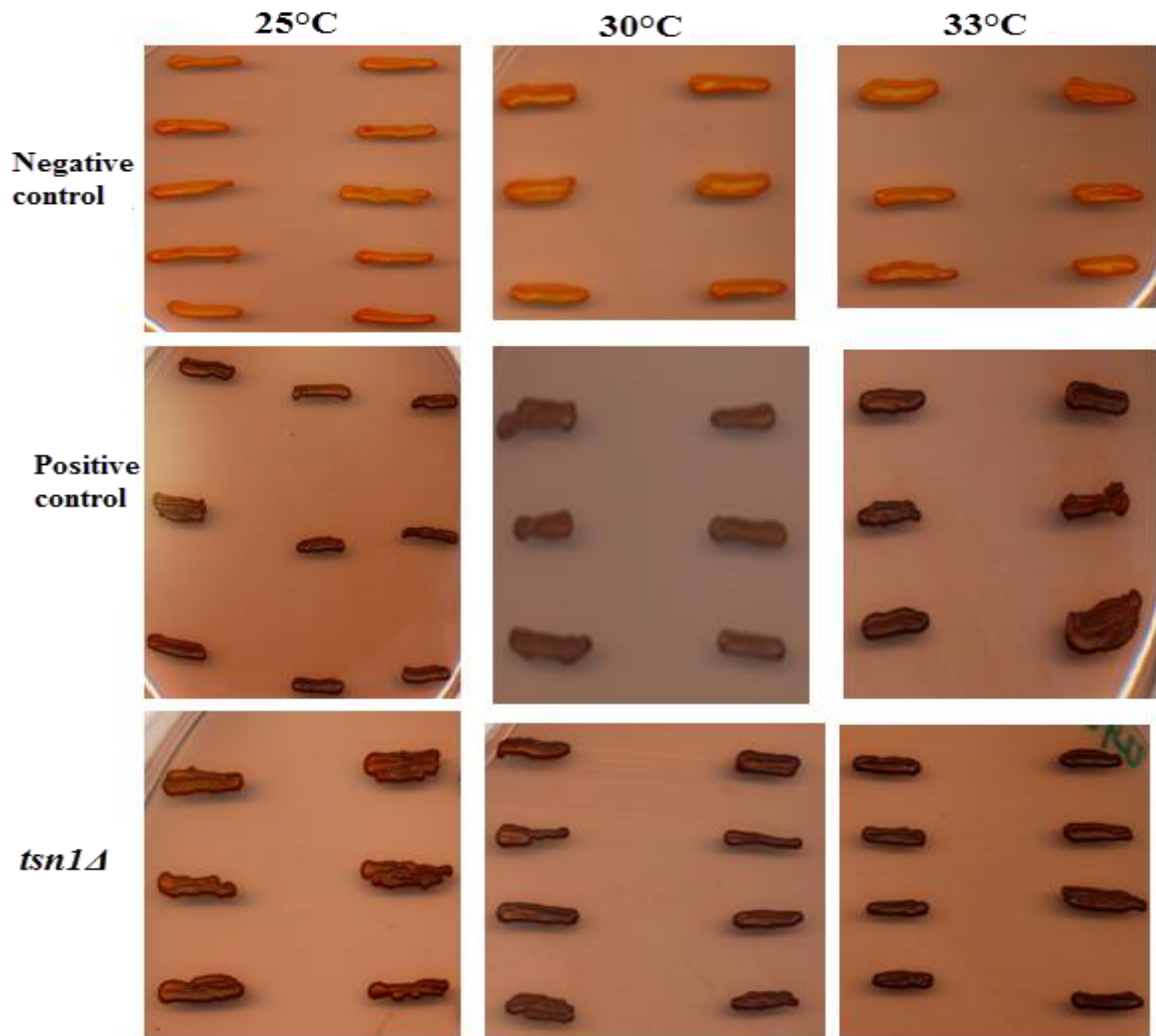


Figure 6.7 mating type iodine staining test in *tsn1Δ*.

Images show mating-type switching capability of *tsn1Δ* deletion strains were colonies sporulated on SPA media and allowed to grow at 25°C, 30°C or 33°C for 3 days then staining with iodine vapours to demonstrate the starch containing spores.

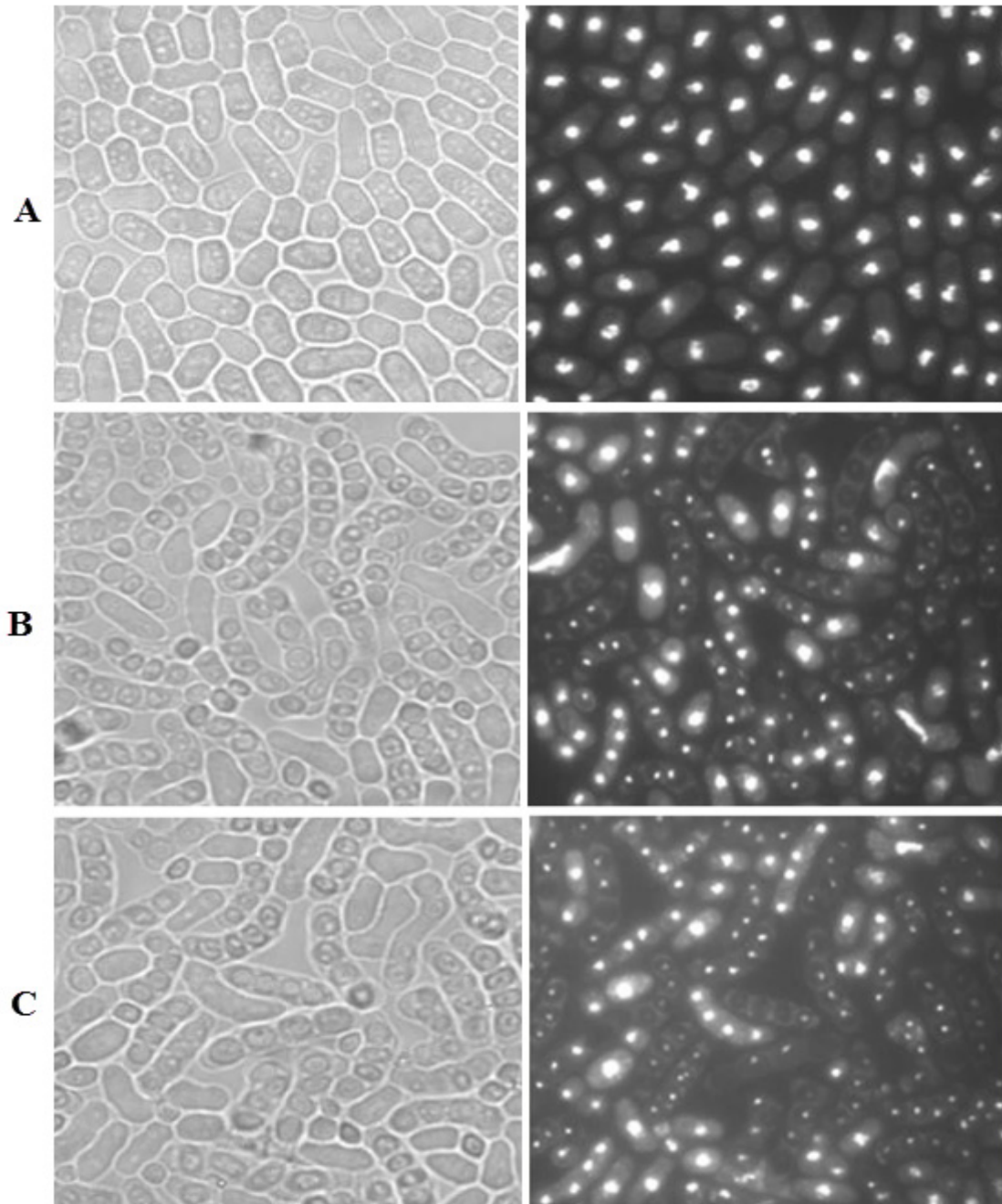


Figure 6.8 Spore morphology.

A. Cell morphology of wild-type haploic cells where no asci form. B. Spores morphology of positive control h^{90} showing 4 spore asci. C. Asci morphology of $tsn1\Delta$. Wild-type, h^{90} strains and mutants strain were incubated on sporulation media to induce mating and sporulation at 30°C for 3 days and spores, fixed, stained with DAPI (right) and examined by microscope.

6.3. Discussion

6.3.1 No effect of double mutants on *rDNA* gene silencing.

rDNA are clustered as families of long tandem head-to-tail repeats and located at specialised heterochromatic region reporter genes silenced (Coulon *et al.*, 2004; Kobayashi *et al.*, 2004; Cam *et al.*, 2005). The resulting strains that compare the growth assay do not show any significant difference on media supplement with 5-FOA indicating that Translin does not contribute to rDNA gene silencing. Furthermore, this indicates the genome instability obtained in the *tsn1Δ dcr1Δ* mutant (Chapter 4) is not due to dysfunction rDNA heterochromatin, which could have resulted in instability of this region of chromosome III.

6.3.2 Silencing at the *mat* locus.

The mating-type loci of *S. pombe* provide a good system to test how heterochromatic histone modifications affect gene expression (Hansen *et al.*, 2011). *mat2-P* and *mat3-M* are firmly silenced by heterochromatin in wild-type cells (Hansen *et al.*, 2011). The double mutants strains do not exhibit any negative effect on *mat* gene silencing. Indeed, none of the mutants tested exhibited loss of gene silencing, including *ago1Δ* and *dcr1Δ*. It is known that heterochromatin-mediated silencing at the *mat* locus can be generated by two redundant pathways. One is dependent on the RNAi machinery, the other requires the stress activated ATF/CREB pathway (Jai *et al.*; 2004). The lack of measurable *mat* gene silencing in the *ago1Δ* and *dcr1Δ* mutants is likely to be due to the activity of this pathway. The fact that the *tsn1Δ ago1Δ/dcr1Δ* double mutants do not alleviate silencing indicates Translin is not likely to be working in the ATF/CREB, RNAi-independent pathway. These observations also relate to mating switching as well as *mat* gene silencing, as no defect in asci frequency or morphology was observed in the *tsn1Δ*.

6.3.3 Less sensitive phenotype in subtelomeric heterochromatin in a double mutants (*tsn1Δago1Δ*).

Telomeres are specialised DNA proteins and protecting structures, which protecting the chromosomes from end-to-end fusions. Genes inserted adjacent to telomeres are transcriptionally repressed because distribution of silent heterochromatin (Bah, and Azzalin, 2012). Therefore heterochromatin is implicated in telomere integrity (Bisht *et al.*, 2008). In *S. pombe* this heterochromatin has been reported to form in an RNAi-independent, Taz1-dependent fashion (Kano et al, 2005). Whilst our analysis reverted no role for Translin in gene silencing at either sub-telomeric region, the different requirement for *ago1* and *dcr1* are remarkable. When Kano and co-workers (2005) concluded that the RNAi machinery played no role in sub-telomeric heterochromatin formation, they did so base on the study of *dcr1Δ* single mutants. Here we show *ago1Δ* to behave similarity to wild-type, what is remarkable is the fact the *dcr1* exhibits an enhanced silencing relative to both wild-type and *ago1Δ*. This suggest that *dcr1* inhibits silencing by another pathway, possibly a Taz1-dependent pathway as loss of *dcr1Δ* increased silencing. This exciting observations is outside the remit of this current story, but is worthy of future investigation.

Conclusions

- 1- Translin plays no measurable role in controlling gene silencing at the *mat* , *rDNA* and sub-telomeric heterochromatic regions
- 2- *dcr1* suppresses extensive gene silencing at the 30 kbp sub-telomeric locus, *ago1* does not.

Chapter 7 Final discussion

7.1 Introduction

Since the initial discovery of Translin in separate studies in the mouse and humans, the identification of specific biological and biochemical functions for Translin and Trax has remained persistently difficult, resulting in fragmented sets of evidence for a functional role in a diverse array of biological functions (Jaendling & McFarlane, 2010). This functional diversity was apparent from the very start of studies with Translin, as it was initially implicated in brain RNA metabolism in the mouse and genome stability regulation in humans (Han *et al.*, 1995; Aoki *et al.*, 1995). In more recent times, during the course of the work reported within this thesis, studies have reported that Translin and Trax function together as key regulators of a RNA metabolism, playing a central role in the RNAi pathway and in tRNA processing (Li *et al.*, 2012). This has resulted in a new dogma with the proposal that the diverse biological functions in which these proteins are implicated are all due to general defects caused by the partial failure in fundamental RNA processing, either RNAi and/or tRNA processing (Liu *et al.*, 2009; Li *et al.*, 2012). However, whilst this seems a sensible and comforting suggestion at first viewing, it does not seem to hold up to deeper scrutiny and the work presented here challenges at least one of the central pillars of this new dogma, suggesting that the biology of these two highly conserved proteins is rather more complex than the simple model would have us believe. Here evidence is presented to demonstrate that loss of Translin function in the fission yeast does not result in wide scale phenotypic abnormalities. Indeed, loss of Translin function alone appears to have no as yet measured negative effect on fission yeast cells. Secondly, the additional genome instability we observe in cells defective for Dicer and Translin functions indicate that Translin functions in an auxiliary pathway to maintain chromosomal balance

when the RNAi pathway is de-regulated. This is not due to a general defect in genome maintenance pathways, suggesting that this is unlikely to be due to a general cellular defect arising from failures to correctly process tRNAs. This core finding is discussed in further detail below.

7.2 Translin provides an auxiliary pathway for genome maintenance in absence of

Dicer

Previously Translin has been implicated in genome stability regulation, and indeed, the finding that human Translin binds to chromosomal breakpoint junctions in cancers leads to the initial suggestion that Translin was a key regulator in an undefined chromosome maintenance pathway. This was supported by additional evidence pointing to a role in DNA damage response and the finding that Translin had DNA binding activity (Jaendling & McFarlane, 2010). Additionally, Translin is capable of forming a toroid, a structure often associated with DNA repair proteins (Jaendling & McFarlane, 2010). These tentative lines of evidence lead to the use of the fission yeast to explore whether Translin was required as a primary regulator of the DNA damage response. Studies using a wide range of DNA damaging agents indicated that this was not the case and there was no direct evidence that fission yeast Translin was required for any DNA repair pathways (Jaendling et al., 2008).

Early during the course of this current study Li and co-workers demonstrated that Translin and Trax make up the C3PO complex, and enhancer of the function required to remove the passenger strand during Argonaute-mediated gene silencing in *Drosophila*. This led us to speculate that Translin might function to direct the RNAi pathway in the fission yeast.

However, mutation of most RNAi pathway regulators results in deregulated heterochromatin at the centromeres, which confers sensitivity to TBZ, the microtubule destabilizing agent. Previous work from the McFarlane group had demonstrated that *tsn1Δ* single mutants did not have this phenotype (Jaendling *et al.*, 2008). In this current work this hypothesis was further challenged by studying the centromeric gene silencing in *tsn1Δ* single mutants, as it might be the case that a subtle defect in centromere heterochromatin regulation did not result in measurable TBZ sensitivity. This was not the case (Chapter 3). Following this, we hypothesised that Translin may have a function that is redundant with one or more of the RNAi regulators. Previous work had demonstrated that a *dcr1Δ* null mutant was less defective than a specific *drc1* mutation in gene silencing (Emmerth *et al.*, 2010). This suggested that the *dcr1Δ* mutant was not fully defective in all gene silencing and that other factors must regulate the residual centromeric gene silencing (Emmerth *et al.*, 2010). In addition, Halic and Moazed (2010) reported a possible Ago1-dependent, Drc1-independent pathway that utilises primary RNAs to trigger heterochromatin function. Translin, like Dicer, has been reported to be associated with an RNase activity (Wang *et al.*, 2004). We speculated that Translin might provide a residual and redundant function with Dicer in the processing of RNAs involved in centromeric heterochromatin-mediated gene silencing. To test this we generated double mutants of *tsn1Δ dcr1 Δ* and *tsn1 Δ ago1 Δ*. On testing these viable mutants the *tsn1 Δ dcr1 Δ* double mutant was found to be more sensitive to TBZ than either single mutant and had a higher frequency of mini chromosome loss than either single mutant. Surprisingly, our initial analysis indicated that this was not due to any additional defect in the levels of centromeric heterochromatin as measured by gene silencing assays (Chapter 5) (however, see below). Given the lack of a defect in heterochromatin-mediated gene silencing at any heterochromatic loci, we tested whether the increased genome instability in the double mutant could be due to a DNA repair defect.

The double mutant showed no significant sensitivity to any of the agents we tested it against, suggesting the chromosome instability phenomena is not due to a DNA repair defect. At this stage of this study it was uncertain which pathway has been compromised by loss of Translin function. However, this is the first evidence in fission yeast of a functional role in genome stability regulation for Translin. Despite the implication of human Translin being involved in break point recombination in cancer-associated translocation formation, we find no evidence to support a role in a recombination mechanism as we observe no sensitivity in single or double mutants to chromosomal breaking or DNA replication pausing agents. Additionally, fission yeast Translin has a higher biochemical affinity for RNA than DNA (Jaendling & McFarlane, 2010), pointing more to a possible role in RNA metabolism. The fact that the activity of Dicer is relatively well documented it is not unreasonable to suggest that Translin plays a role in some aspect of RNA metabolism which is normally largely executed by Dicer, which is independent of mediating heterochromatin-mediated gene silencing, but does impinge upon genome stability.

7.2 A role in microtubule dynamics

Previously Translin function has been implicated in microtubule dynamics. In this study we have largely failed to address this question as we prioritised a hypothesis which postulated that Translin was involved in RNAi-mediated centromere function. However, it remains a formal possibility that Translin does have a role in microtubule dynamics. This can only be a minor role as any defect caused by loss of Translin does not manifest a phenotype in a *tsn1*Δ single mutant and is dependent upon additional loss of Dicer. Given this we believe that this is an unlikely explanation for the increased genome instability observed in the double mutant. It could be possible that a minor microtubule defect is only

apparent when centromere function becomes compromised by loss of Dicer, but one might have expected the loss of Argonaute to also result in a centromere defect which exposed this hypothetical microtubule defect. This was not the case as the *ago1Δtsn1Δ* mutants have similar mini chromosome loss and TBZ sensitivity as the *ago1Δ* strain. Whilst we are dismissive of the possibility of a role in microtubule dynamics, the *ago1Δ tsn1Δ* data are not sufficient to totally reject this possibility. This should be tested further by directly measuring microtubule activity. At the simplest level microtubule structures can be measured following immunofluorescence to determine whether their size and morphology is different in the *tsn1Δ* cells. More involved experimentation might be aimed at determining microtubule dynamics and the kinetics of activity during the cell cycle using time lapse microscopy in live cells using GFP-labelled microtubules. In addition studies on the interaction of microtubules with other macromolecules might reveal a defect in their ability to modulate other cell division related processes. Unfortunately, these proposals were beyond the remit of this current study.

7.3 Could translin function solely in tRNA processing?

Lui and co-workers (2012) recently reported a role for Translin and Trax in tRNA processing. This work was only published relatively late within the time period of this work. However, in this study Lui and co-workers (2012) propose that general defects are due to a general defect in protein production arising from a failure to correctly process all tRNAs. This could be the case for fission yeast Translin, but, as indicated above, we believe this to be unlikely as a genome maintenance deregulation is only apparent in a *dicer* defective background, indicating a highly specific loss of function. We have examined a range of fundamental biological processes in *tsn1Δ* single and *tsn1Δ dcr1Δ/ago1Δ* double mutants and find a very specific defect (loss of chromosome stability) in one genetic background. To us, this points to a very specific and specialist function for Translin, one which is an auxiliary role to that normally carried out by Dicer. If this role were a specialised processing of a sub-set of functionally specific tRNAs, then the finding that *dicer* functions as the predominant regulator would be a remarkable one and certainly merits further exploration. Moreover, our data might suggest that whilst this is Dicer-dependent, there is no evidence that it is Argonaute-dependent. Interestingly, Lui and co-workers reported that there were other RNA species that were affected by the loss of Translin function, although there was no commonality in function or features for these RNAs. It might be the case that Translin targets a specific group of RNAs and that in different species Translin has evolved to play specific roles relating to the function of a defined sub-group of RNA molecules. This argument is supported by the observation that Translin binding sequences are found within the coding region of the *Brain Derived Neurogenic Factor (BDNF)* mRNA in humans and that Translin is required for the correct localisation of this mRNA in the brain (Chiaruttini *et al.*, 2009). This indicates that the Translin story is more complex than a simple processor of tRNA precursors. Indeed, there might be diverse species and tissue specific activities for Translin which, whilst remaining

conserved in biochemical activity (and hence sequence) has diverged in target specificity resulting in the broad range of biological functions Translin has been associated with. To take this hypothesis further in the fission yeast makes some sense, as it is amenable to work to discern how a specific set of RNA molecules might control genome stability. RNA sequencing of *tsn1Δ* and *tsn1Δ dcr1Δ* whole transcriptomes might provide solid evidence for which, if any RNA molecules are targeted by Translin in fission yeast, and, more importantly, what specific aspect of genome stability this group of RNAs regulate. Moreover, what is the role of dicer and how does this relate to the role dicer plays in controlling the RNAi pathways.

7.4. A final answer

During the course of this work one of the key surprises was that we did not observe any increased loss of gene silencing of centromeric heterochromatin in the *tsn1Δ dcr1Δ* double mutant relative to the single mutant (see above). Halic and Moazed (2010) had demonstrated that the epigenetic mark of heterochromatin, methylation of histone H3 on residue lysine 9 (H3K9me2), was not reduced in a *dcr1Δ* mutant to the same degree as it was in the *ago1Δ* mutant. From this, and other observations, they proposed a model for heterochromatin in which an Ago1-dependent, Dcr1-independent pathway generated heterochromatin utilising primary RNAs (Figure 7.1). They noted that the primary pathway was Dcr1-dependent and that this other pathway was secondary to this (Figure 7.1). Consistent with this, Emmerth *et al.*, (2010) showed that a specific *dcr1* truncation mutant (*dcr1-Δ33*) exhibited a greater loss of pericentric gene silencing than a *dcr1Δ* null mutant, indicating that this truncation mutant had a dominant negative effect suggesting an additional Dcr1-independent pathway, supporting the proposal of Halic and Moazed (2010) (Figure 7.1).

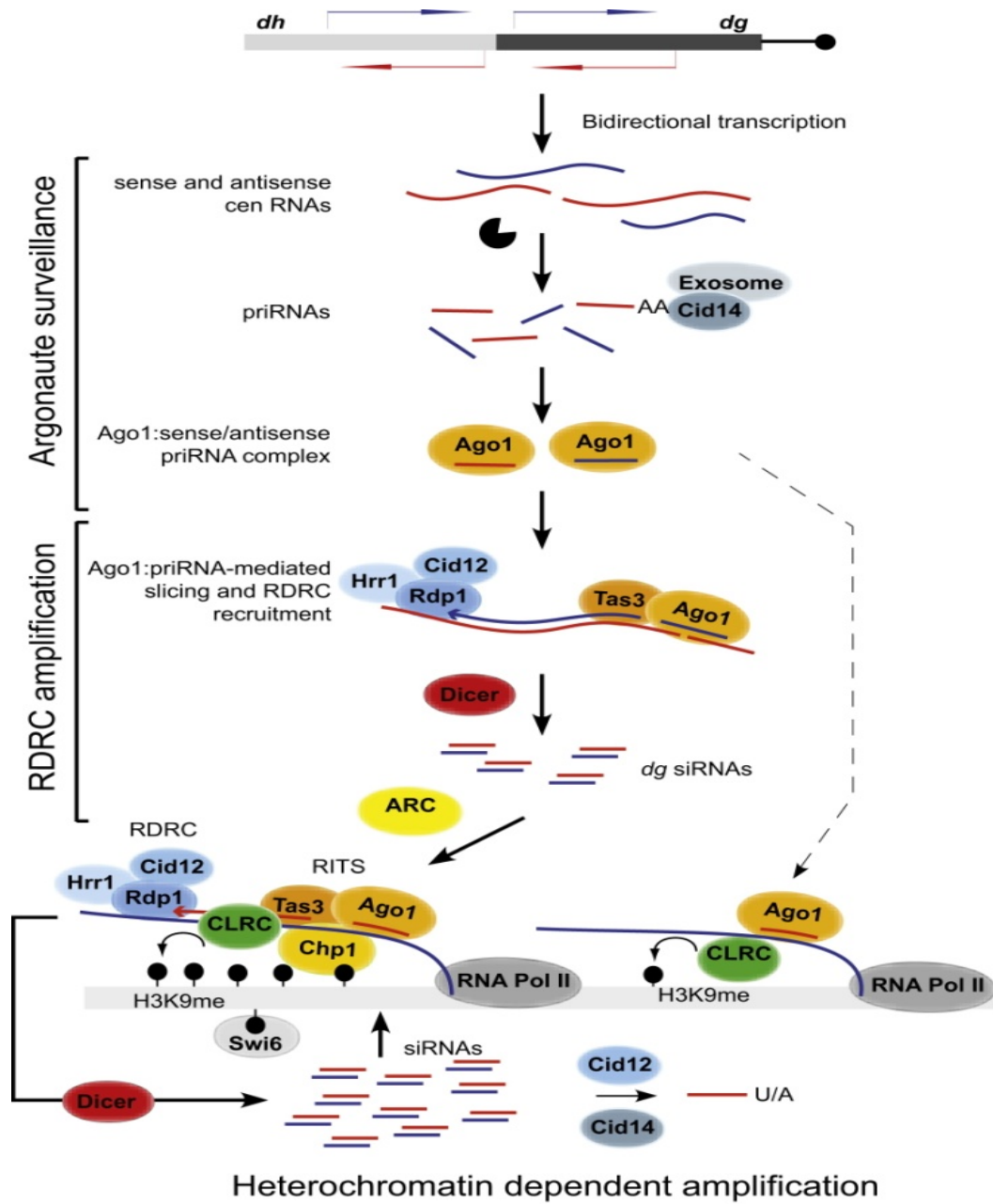


Figure 7.1. Halic and Moazed model for an Ago1-dependent, Drc1-independent pathway for the maintenance of H3K9me₂-dependent centromeric repeat (*dh dg*) heterochromatin in fission yeast. The primary route to form heterochromatin depends on exosome-generated primal small RNAs (priRNAs) being acted upon by Argonaute (Ago1) to ultimately recruit the RNA-dependent RNA polymerase complex (RDRC). In turn, via the action of Dicer (Dcr1)-generated *dg* small interfering RNAs (siRNAs), this results in the RNA-induced transcriptional silencing (RITS) complex, driving the methylation of H3K9 by the Clr4-Rik1-Cul4 (CLRC) complex which forms the binding sites for Swi6, the fission yeast orthologue of Heterochromatin Protein 1. The auxiliary pathway (dashed line, right hand side) is dependent upon priRNAs and Ago1, but is independent of Dicer. The factors driving the auxiliary pathway were not known. Figure taken from Halic & Moazed (2010).

These findings forced us to re-visit our analyses of the centromeric gene silencing data we had obtained. In the light of finding no other defect in the *tsn1Δ dcr1Δ* strains which could account for the chromosomal instability, we decided to re-visit the hypothesis that Translin was responsible for the secondary pathway for maintaining centromeric heterochromatin. To this end we repeated the gene silencing assays carried out in Chapter 5. In these assays cells expressing the *ura4⁺* gene which is embedded in the centromeric heterochromatin become sensitive to 5-FOA. In our original experiment we had employed a standard concentration of 5-FOA. However, we postulated that this standard concentration was too high, potentially masking any differences in gene expression between the *dcr1Δ* and the *tsn1Δ dcr1Δ* mutants. To address this, the experiment was repeated employing lower 5-FOA concentrations (400 µg /ml). The results were clear and striking (Figure 7.2). At these lower 5-FOA concentrations it can be seen that the *dcr1Δ* mutant is not as sensitive to 5-FOA as the *ago1Δ* mutant. This is consistent with the Halic and Moazed (2010) model (Figure 7.1). Moreover, the *tsn1Δ dcr1Δ* double mutant exhibits a sensitivity to 5-FOA which is lower than the *dcr1Δ* single mutant and the same as the *ago1Δ* mutant. These data reveal a very clear answer to why we get enhanced chromosomal instability in the *dcr1Δ* mutant when *tsn1* is also mutated, as centromeric heterochromatin is clearly more defective.

From these final findings we can clearly postulate that Translin functions in the secondary Ago1-dependent, Dcr1-independent pathway for centromeric heterochromatin maintenance and centromere function (Figure 7.3). Exactly what functional role Translin fulfils remains unknown; moreover, it is unclear whether this is dependent upon the RNase activity of Translin. These findings could be construed to be consistent with a model in which the conserved function of Translin is in an RNAi-related pathway, and is inconsistent with a conserved function in tRNA processing which results in general cellular defects. However, whilst we have no data to indicate a role in tRNA processing in fission yeast, and we can

dismiss a generalised defect in cellular activity, we cannot dismiss specific additional functional roles for Translin in processing of tRNAs and/or other RNAs, other than to suggest that if such functions do exist, they too are likely to be redundant with other cellular processes, possibly Drc1-dependent processes.

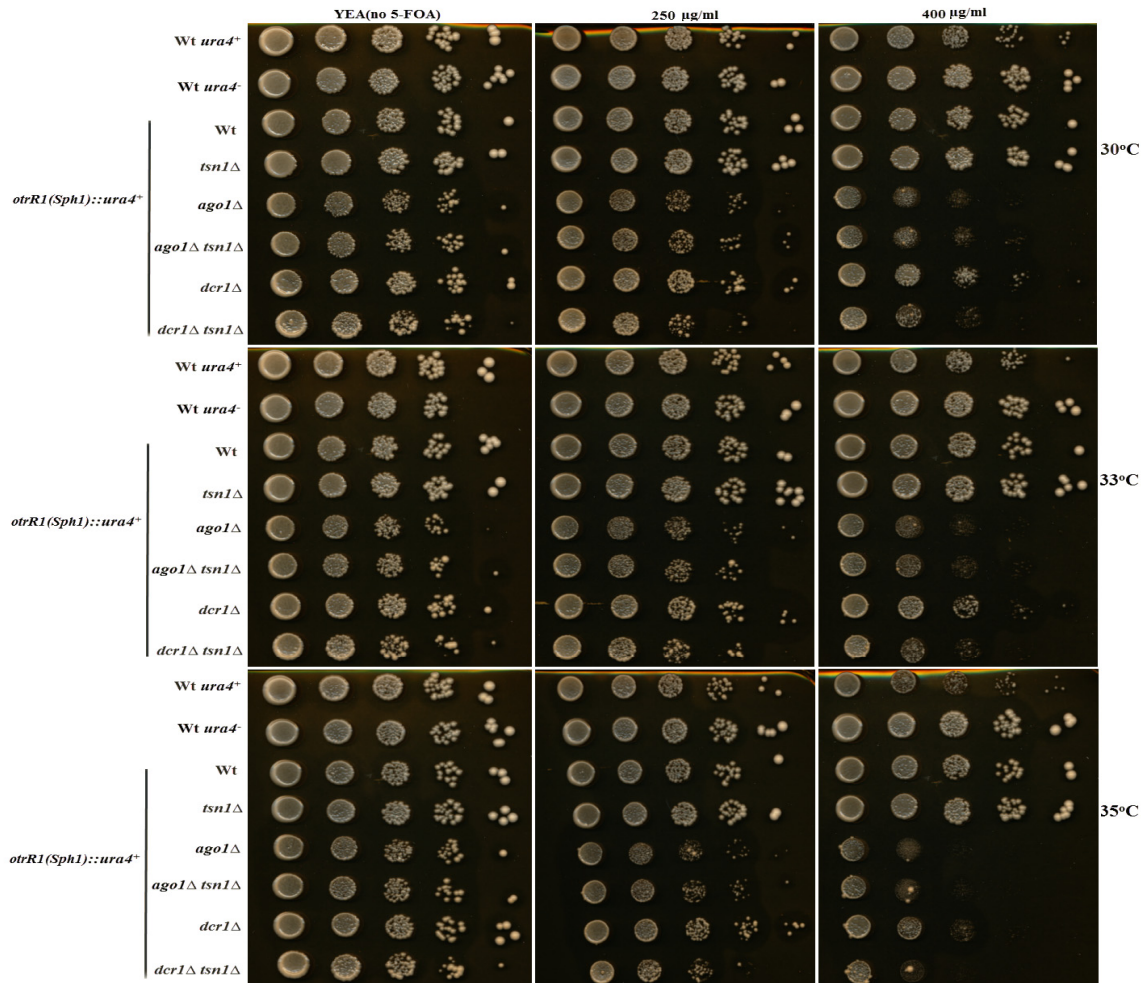


Figure 7.2. Translin regulates heterochromatic gene silencing in the outer repeats (*otr*) of fission yeast centromeres in a Dicer-independent fashion. Serial dilutions of various cells were spotted onto plates containing either 250 µg/ml or 400 µg/ml 5-FOA (two right hand panels). Wild-type and *tsn1Δ* strains exhibit no loss of centromeric silencing (5-FOA sensitivity). The *dcr1Δ* mutant exhibits increased of silencing function than the *ago1Δ*, the *ago1Δ tsn1Δ* double mutant or the *drc1Δ tsn1Δ* double mutant at all temperatures, indicating that Translin (Tsn1) serves in a redundant pathway to mediate centromeric heterochromatin-mediated gene silencing.

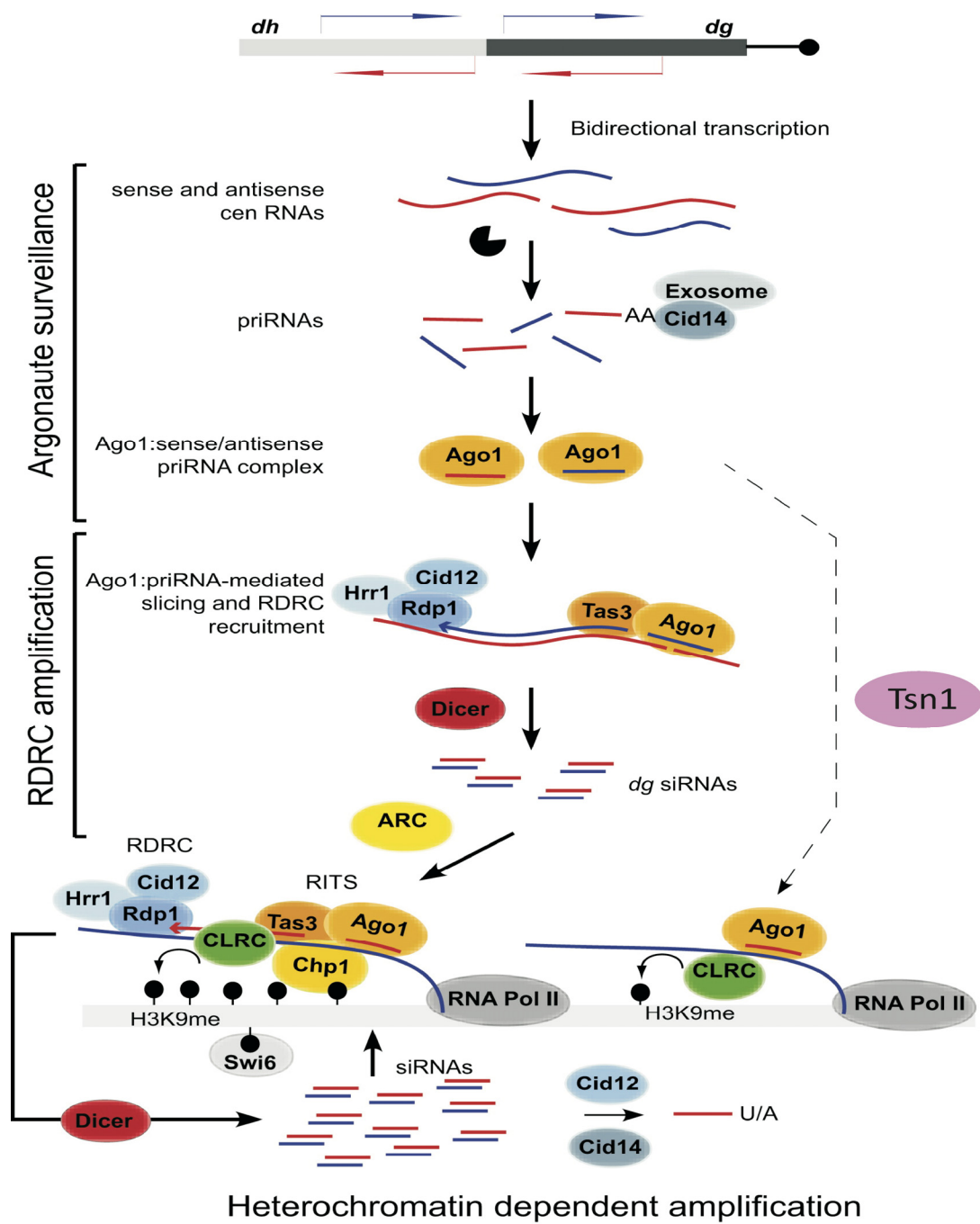


Figure 7.3. Translin functions in an Ago1-dependent, Dcr1-independent pathway for centromeric heterochromatin maintenance. The Ago1-dependent, Dcr1-independent pathway proposed by Halic and Moazed is proposed to be mediated by Translin (Tsn1) (refer to Figure 7.1 for more details). Adapted from Halic and Moazed (2010).

Interestingly, *tRNA* genes are required to serve as heterochromatin boundary functions within the fission yeast (McFarlane & Whitehall, 2009). It remains possible that these centromere *tRNA* genes require Translin for their expression / product processing and that this is associated with boundary function and thus heterochromatin structure. This intriguing possibility would link all the observations made by us and others enabling a unified model to be presented for the function of Translin. The fission yeast offers an excellent model system to test this hypothesis in the future.

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